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14. ABSTRACT
The overall goal of this proposal is to develop fully synthetic vaccines against respiratory infections using novel nanotechnology platforms based on safe and degradable adjuvant polymer systems that enhance antigen presentation and stimulate immunity. The proposal focuses on achieving the following specific technical objectives: 1) Develop molecular methods for intervention strategies employing novel synthetic nanovaccine platforms encapsulating DNA and protein/peptide antigens that elicit immune response against influenza H5N1; 2) Test the efficacy of nanovaccines-based intervention regimens against influenza H5N1 in animal models. 3) Adopt intervention strategies using most promising nanovaccine platforms to other respiratory infections, such as pulmonary F. tularemia, and test the nanovaccines efficacy in animal models (unfunded option). Focusing on respiratory infections, the leading cause of outpatient illness and a major cause of infectious disease hospitalization in U.S. military personnel, has the potential to develop effective, safe and affordable synthetic vaccines.

15. SUBJECT TERMS
Synthetic vaccines against respiratory pathogens

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INTRODUCTION

The overall goal of this proposal is to develop fully synthetic vaccines against respiratory infections using novel nanotechnology platforms based on safe and degradable adjuvant polymer systems that enhance antigen presentation and stimulate immunity. Three novel versatile technology platforms will be evaluated: a) biodegradable polyanhydride nanospheres (BPN) carrying a polypeptide/protein antigen (Platform A); b) polymeric micelles of Pluronic block copolymer as DNA vaccine adjuvant (Platform B); and c) block ionomer complexes (BIC) for targeted delivery of DNA (or protein) antigen to the antigen presenting cells (APCs) (Platform C). The central hypothesis is that these polymeric nanoscale delivery systems can provide versatile platforms for development of effective, safe, and cost-efficient vaccines. The materials used in these platforms demonstrated immunomodulatory capabilities and can be targeted to specific populations of immune cells to elicit most efficient immune response. The best technology or their combination will be determined for future development.

The proposal is focusing on achieving the following specific technical objectives: 1) Develop molecular methods for intervention strategies employing novel synthetic nanovaccine platforms encapsulating DNA and protein/peptide antigens that elicit immune response against influenza H5N1; 2) Test the efficacy of nanovaccines-based intervention regimens against influenza H5N1 in animal models. 3) Adopt intervention strategies using most promising nanovaccine platforms to other respiratory infections, such as pulmonary *F. tularemia*, and test the nanovaccines efficacy in animal models (unfunded option).

BODY OF REPORT

We are on target or ahead in most of the tasks as described in two previous annual reports (for YEAR 1 and 2) and further accounted for in the present annual report (YEAR 3). The following tasks and timeline were proposed:

YEAR 1
Task 1: Establish project: a) the project teams will be assembled at both sites of project performance; b) new essential equipment will be acquired; and c) IACUC and IBC protocols will be submitted and approved. ACCOMPLESHED
Task 2: Produce DNA plasmids expressing viral antigens (Platform A, B, & C).
Task 3: Produce recombinant influenza A virus H5N1 proteins (Platform A & C).
Task 4: Synthesize cationic copolymers for BIC/DNA compositions (Platform C).

YEAR 2
Task 1: Synthesize a library of polyanhydride copolymers (Platform A).
Task 2: Prepare and characterize targeted BIC/DNA compositions (Platform C).
Task 3: Determine optimal Pluronic/DNA and BIC/DNA compositions to maximize levels and duration of transgene expression after administration in skeletal muscle in a mouse (Platform B & C).

YEAR 3
Task 1: Determine optimal antigen-containing BPN that activate dendritic cells (DCs) (Platform A).

Task 2: Use optimal Pluronic/DNA and BIC/DNA compositions determined in Y02 to achieve maximal antigen expression (Platform B & C).
Task 3: Establish/adapt model for aerosol virus challenge; test viability/infectivity of virus preparations.
Task 4: Determine optimal antibody response generated by nanovaccine developed in each platform.

YEAR 4
Task 1: Evaluate vaccines, assess immunogenicity in vivo, and determine the lead nanovaccine configurations for aerosol virus challenge studies.
Task 2: Determine vaccine that protect against H5N1influenza virus in challenge studies.

Platform A

To rationally determine the optimal polyanhydride chemistries to release stable hemagglutinin (HA) a full-length HA H5 protein (Boehringer Ingelheim Vetmedica) was incubated in phosphate buffered saline (PBS) with varying pH and temperature. By studying the tertiary structure of HA over a period of one week it was shown the protein is more stable in mild acidic conditions versus normal pH (Figure 1).

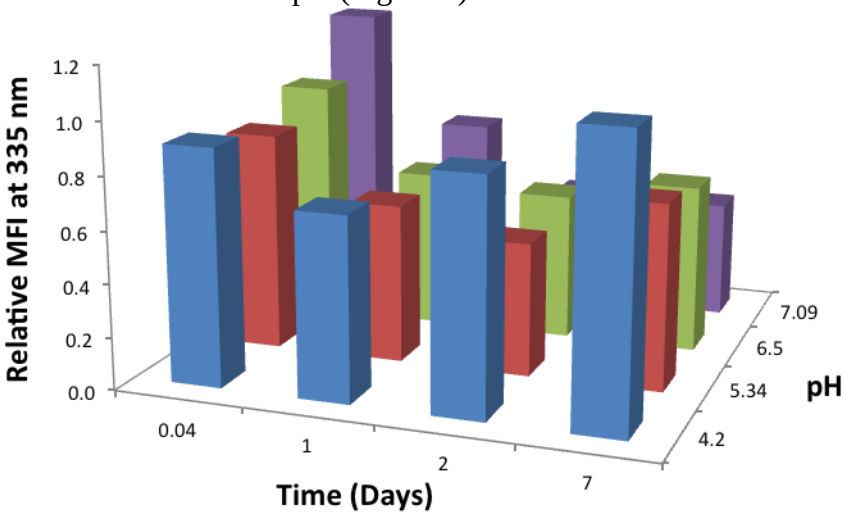


Figure 1: Tertiary structure of HA shows increased stability under acidic pH. Mean fluorescence intensity (MFI) of the HA maximum peak (335 nm) shows increased stability under acidic conditions. HA incubated at neutral pH shows deterioration of the protein's tertiary structure.

Polyanhydride nanoparticles containing two percent HA were synthesized via a solid/oil/oil method to determine protein stability and release kinetics from nanoparticles. Three polyanhydride copolymers were chosen based on the pH of their degradation products (4.5-5.5) to stabilize HA: 20:80 CPH:SA, 20:80 CPTEG:CPH, and 50:50 CPTEG:CPH. Briefly, the polymer was dissolved in chilled methylene chloride at a concentration of 20 mg/mL with 2% HA protein. The solution was sonicated for approximately 30 s and = poured into pentane chilled with liquid nitrogen at a solvent:non-solvent ratio of 1:200. Protein-loaded particles were collected via vacuum filtration and characterized by scanning electron microscopy (Figure 2).

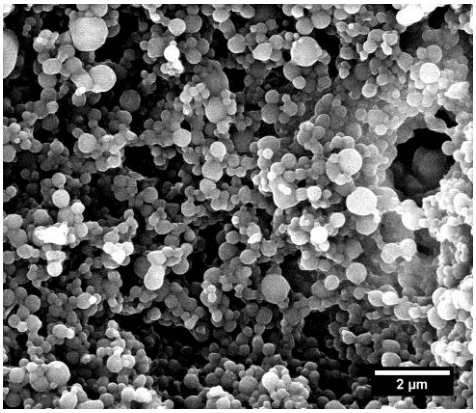


Figure 2: SEM of 2% HA nanoparticles. Scanning electron microscopy image displaying the approximate size and morphology of 2% HA 20:80 CPH:SA nanoparticles.

The nanoparticles were incubated in PBS and sampled for protein release for approximately one month. Encapsulation of HA into polyanhydride particles was found to sustain release of the protein with zero order kinetics (Figure 3), consistent with our previous work utilizing other proteins. Structural studies also shown the primary (SDS PAGE) and tertiary structure (fluorescent spectroscopy) structure of the protein was maintained upon release. The tertiary structure of released protein showed an increase in mean fluorescent intensity, which may indicate the exposure of buried fluorescent residues during conformational changes upon release (Figure 4). Noting that the pH of polyanhydrides corresponds to the conformational change of HA, shifts in the peak emission wavelength show conformational changes and increased stability of released HA consistent with previous experiments.

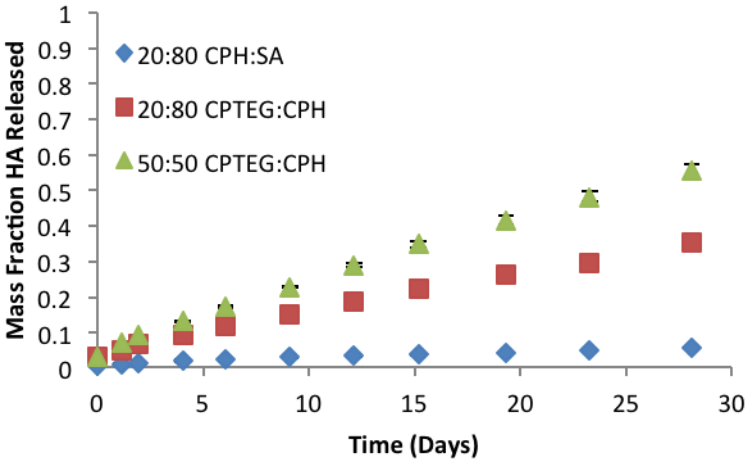


Figure 3: Release kinetics of HA. Polyanhydride nanoparticles showed zero order release kinetics of HA over approximately one month. As expected, more amphiphilic copolymers (CPTEG:CPH) released protein faster than more hydrophobic copolymers (CPH:SA).

With the future intention of observing differences between HA monomer (H5-M) and trimer (H5-T) immunogenicity, as well as cross-protection between H5N1 influenza clades, we

sought to observe the stability of a clade 2.2 H5 HA A/Mongolia/Whooper Swan/244/05 protein synthesized in both monomer and trimer forms. This protein was synthesized by Dr. Wuwei Wu in the laboratories of Dr. Susan Carpenter at ISU.

In vitro work with the recombinant H5 HA A/Mongolia/Whooper Swan/244/05 identified sensitivities of the protein to different buffers as well as processes previously employed for dry powder fabrication into polyanhydride particles. Low solubility due to aggregation and precipitation led to no or minimal detection of the protein in both quantification and structural assays. The solubility of the protein was improved by increasing the pH of buffer (pH 8) further away from the protein's isoelectric point. To improve detection in quantification assays, it was found that a combination of 2% sodium dodecyl sulfate (SDS) and 25 mM sodium hydroxide (NaOH) with HA monomer elicited the most accurate results using a microBCA assay.

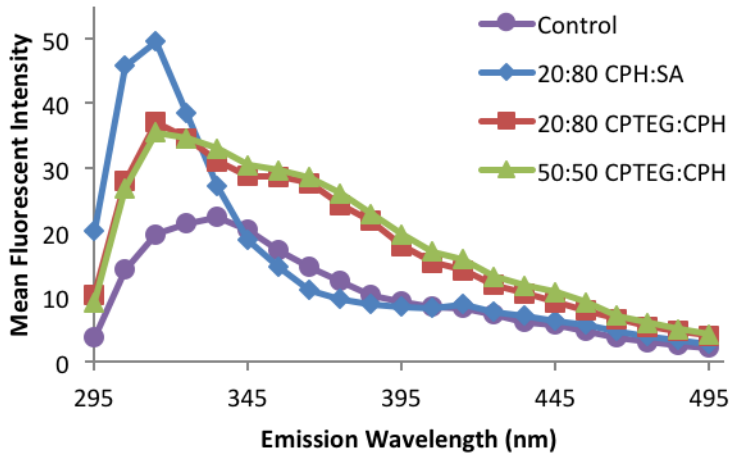


Figure 4: Tertiary structure of released HA. Fluorescent spectroscopy shows the conformational changes and increased stability of HA tertiary structure upon release from polyanhydride nanoparticles.

It was also determined that the lyophilization process led to the instability of the protein. Both the stabilized H5-T and H5-M lose immunogenicity and function when lyophilized to a dry powder form. Soluble HA (sH5), or protein that was never lyophilized, was determined to have increased stability over reconstituted protein. Soluble HA was easily quantified with a microBCA assay, and improved detection was observed in ELISA (antigenicity), SDS PAGE (primary structure), circular dichroism (secondary structure), and fluorescent spectroscopy (tertiary structure) experiments. For example, in Figure 5, lanes loaded with equivalent amounts of protein showed improved detection with soluble HA versus reconstituted HA, even in the presence of additives to increase protein solubility.

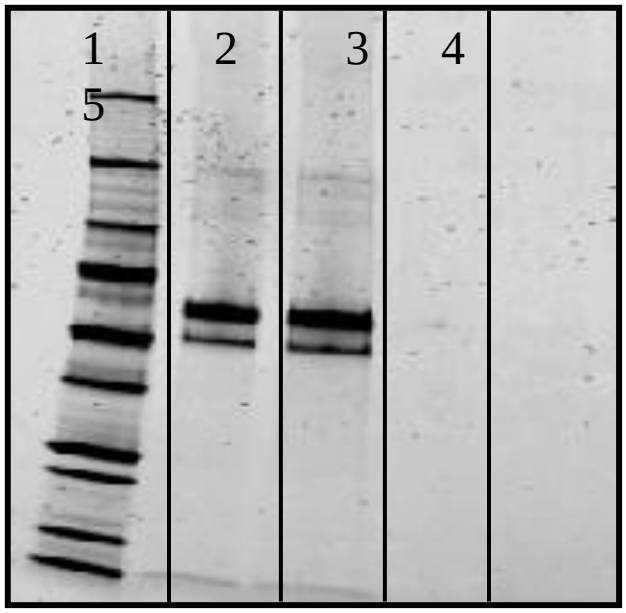


Figure 5: Soluble HA shows increased detection over reconstituted protein in SDS PAGE. SDS PAGE lanes loaded with equivalent amounts of HA protein exhibited darker bands with soluble protein versus reconstituted, even with additives such as SDS and NaOH to increase solubility. Lane assignments: 1) Molecular weight ladder 2) Soluble HA 3) Soluble HA + SDS/NaOH 4) Reconstituted HA and 5) Reconstituted HA + SDS/NaOH.

Although solid/oil/oil nanoprecipitation produced higher encapsulation efficiencies, the fabrication of polyanhydride nanoparticles was modified to a water/oil/oil (w/o/o) method to accommodate encapsulating protein in the more stable, soluble form. Briefly, 500 µg of HA dissolved in water was added to 2.5 mL of methylene chloride containing 50 mg of polymer. The solution was homogenized for 60 seconds and poured into 625 mL pentane. The precipitated nanoparticles were collected via vacuum filtration. Studies are ongoing to determine the encapsulation efficiency of HA in polyanhydride nanoparticles and the stability of HA under particle fabrication conditions.

In parallel to the *in vitro* stability studies, we also performed *in vivo* experiments with the recombinant HA proteins. Our previous work showed that a 10 µg dose of recombinant HA protein was optimal. We also designed a sensitive antibody quantitation platform using Bioplex based methods and technology that allowed for the simultaneous examination of antibody reactivity to both the stabilized trimer and monomer forms of the H5 antigen. This new method enabled the minimization of serum sample and protein usage and resulted in throughput enhancement.

Due to the instability of lyophilized HA, soluble forms of the H5-T were used to immunize BALB/c mice subcutaneously and intranasally (10 µg dose) with and without different traditional adjuvants: monophosphoryl lipid A (MPLA) or aluminum salts (Alum) (Table 1). These studies will be used to benchmark future work with nanoparticle-based adjuvants.

Table 1: Immunization Groups of Soluble H5-T

Group	Antigen	Adjuvant	Route
sH5 SC	10 µg H5-T in PBS	None	Subcutaneous
sH5 IN	10 µg H5-T in PBS	None	Intranasal
MPLA SC	10 µg H5-T in PBS	10 µg MPLA	Subcutaneous
MPLA IN	10 µg H5-T in PBS	10 µg MPLA	Intranasal
Alum SC	10 µg H5-T in PBS	Alum	Subcutaneous
Saline	None	None	Subcutaneous

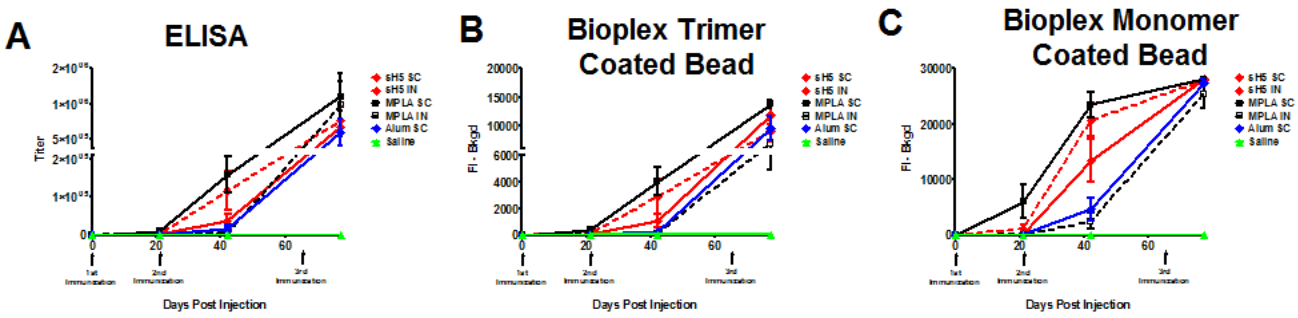


Figure 6: Serum antibody response of mice immunized with soluble H5-T. BALB/c mice immunized with soluble doses of H5-T show increasing amounts of antigen specific antibody after boost.

Weak titers via ELISA or Bioplex antibody quantitation were observed 21 days post a single immunization (Figure 6A-C). A boost immunization was administered at the 21 day post immunization (DPI) timepoint. Serum samples obtained at 42 days post immunization showed increasing amounts of antigen-specific antibody at this time point.

Neutralizing activity against an equine infectious anemia virus expressing the H5 HA A/Mongolia/Whooper Swan/244/05 was observed at the 42 DPI time point, once again pointing to the importance of using soluble protein for vaccine preparations. This was evidenced by the observation of negligible antibody neutralizing activity until this point (Table 2).

Table 2: Neutralization Antibody Titer

Group	Neutralization Titer	
	21 DPI	**42 DPI**
sH5 SC	**<50 ± 0**	**484.78 ± 284.35**
sH5 IN	**<50 ± 0**	**2203.08 ± 1495.09**
MPLA SC	**<50 ± 0**	**5784.01 ± 2523.37**
MPLA IN	**<50 ± 0**	**22.90 ± 21.90**
Alum SC	**<50 ± 0**	**13.06 ± 12.06**
Saline	**<50 ± 0**	**<50**

The highest amount of neutralizing activity was observed in the groups receiving a Toll like receptor (TLR) agonist as an adjuvant. Alum did not show effective adjuvanticity in eliciting neutralizing titer, although all whole protein titers were relatively similar. Neutralization titer data for the 77 DPI timepoint is currently being evaluated. The stability of the protein appears to be of paramount importance in these studies as the acidity due to Alum or crosslinking adsorption may have led to degradation of the neutralizing epitopes of HA. This study also points to the importance of using a prime-boost strategy for eliciting neutralizing antibody from these immunization preparations. As expected, only intranasal immunizations were able to elicit antigen specific IgA antibody in the bronchio-alveolar lavage fluid at the end of the study (Figure 7).

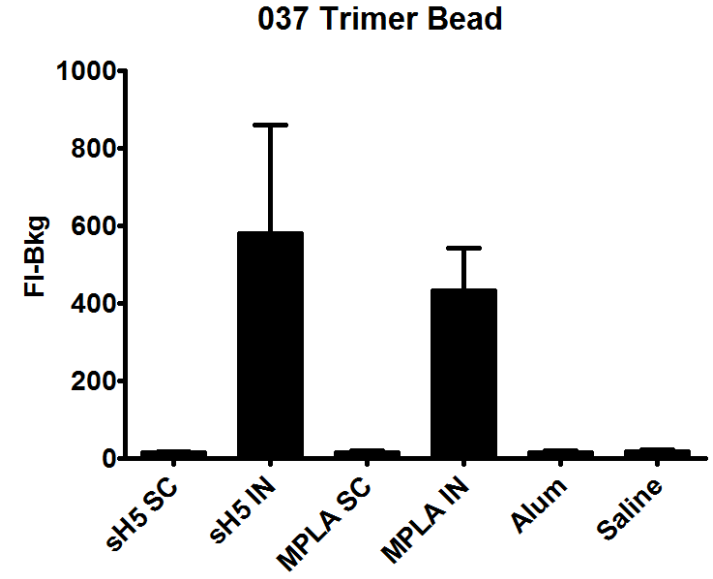


Figure 7: Antigen specific IgA. Intranasal immunizations of soluble HA elicit antigen specific IgA antibody in bronchioalveolar lavage fluid.

Ongoing and future work: Upon optimization of the w/o/o process, the next step is to study the structural and antigenic stability of H5-T and H5-M released from nanoparticles. To complete these studies, a fetuin assay is being developed to test the immunological function of H5-T and H5-M upon release. Fetuin protein, representing the sialic acid residues that bind HA in the lung, will be coated on a high binding, 96-well plate at 1 μg/mL and incubated overnight at 4°C. The following day, each well will be blocked with 300 μL of phosphate buffered saline with 0.05% Tween 20 (PBS-T) and 1% gelatin. After blocking for two hours at room temperature, the plate will be washed with PBS-T and incubated with a monoclonal antibody overnight at 4°C. Finally, results will be determined by alkaline phosphatase absorbance at 405 nm.

The efficacy of single and multiple dose polyanhydride particle-based H5 HA vaccines are currently being planned. The previous data with subcutaneous immunizations of HA protein has led us to initiate studies examining immune responses from subcutaneous immunizations of HA protein encapsulated including encapsulated ligands of TLR's (Poly I:C) and inflammasome (Poly dA:dT) receptors. Previous *in vitro* data with encapsulated Poly I:C showed increased inflammatory cytokine secretion from bone marrow derived macrophages that was dependent on

phagocytic action. The immunization groups for initial polyanhydride nanoparticle-based immunizations are indicated in Table 3. A parallel study examining the efficacy of intranasal immunizations will also be performed with the same groups as shown in Table 3.

Table 3: Immunization Groups for Polyanhydride Nanoparticles

Immunization Group	Abbreviation	Second Immunization
SC-7.5 µg free + 2.5 µg 20:80 CPTEG:CPH	20:80 C:C	Day 21 post injection (p.i.)
SC-7.5 µg free + 2.5 µg 20:80 CPTEG:CPH + 5 µg Poly I:C 20:80 CPTEG:CPH	20:80 C:$C_{poly\ I:C}$	Day 21 p.i.
SC-7.5 µg free + 5 µg Poly I:C + 2.5 µg 20:80 CPTEG:CPH	20:80 C:C + sPoly I:C	Day 21 p.i.
SC-7.5 µg free + 2.5 µg 20:80 CPTEG:CPH + 5 µg Poly dA:dT 50:50 CPTEG:CPH	20:80 C:$C_{poly\ dA:dT}$	Day 21 p.i.
SC-7.5 µg free + 5 µg Poly dA:dT + 2.5 µg 20:80 CPTEG:CPH	20:80 C:C + sPoly dA:dT	Day 21 p.i.
10 µg MPLA x 2	MPLA	Day 21 p.i.
10 µg soluble H5 Trimer	sH5T	Day 21 p.i.
Saline	Saline	None

In vitro T cell proliferation assays are also being developed to examine the ability of our polyanhydride nanoparticle immunization platform to activate macrophages and the subsequent antigen presenting capabilities of these macrophages using transgenic T cells specific for outlined peptide sequences.

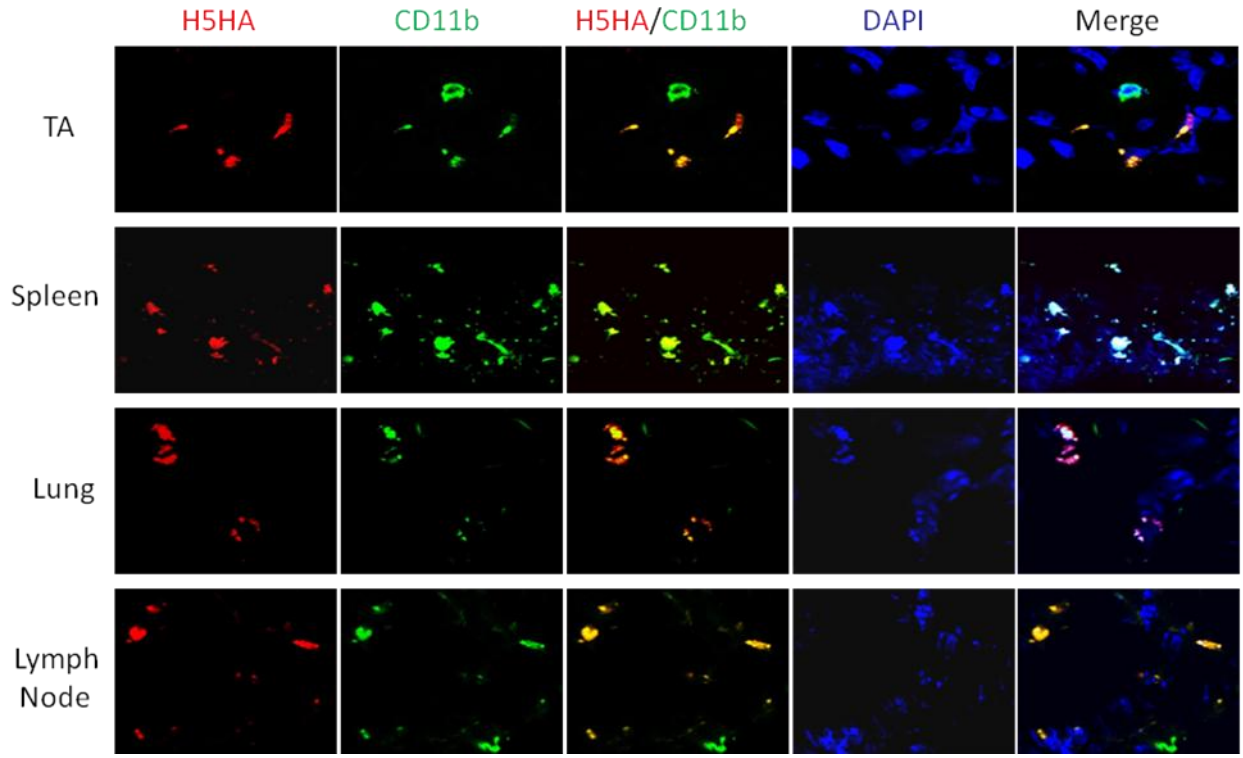
Platform B

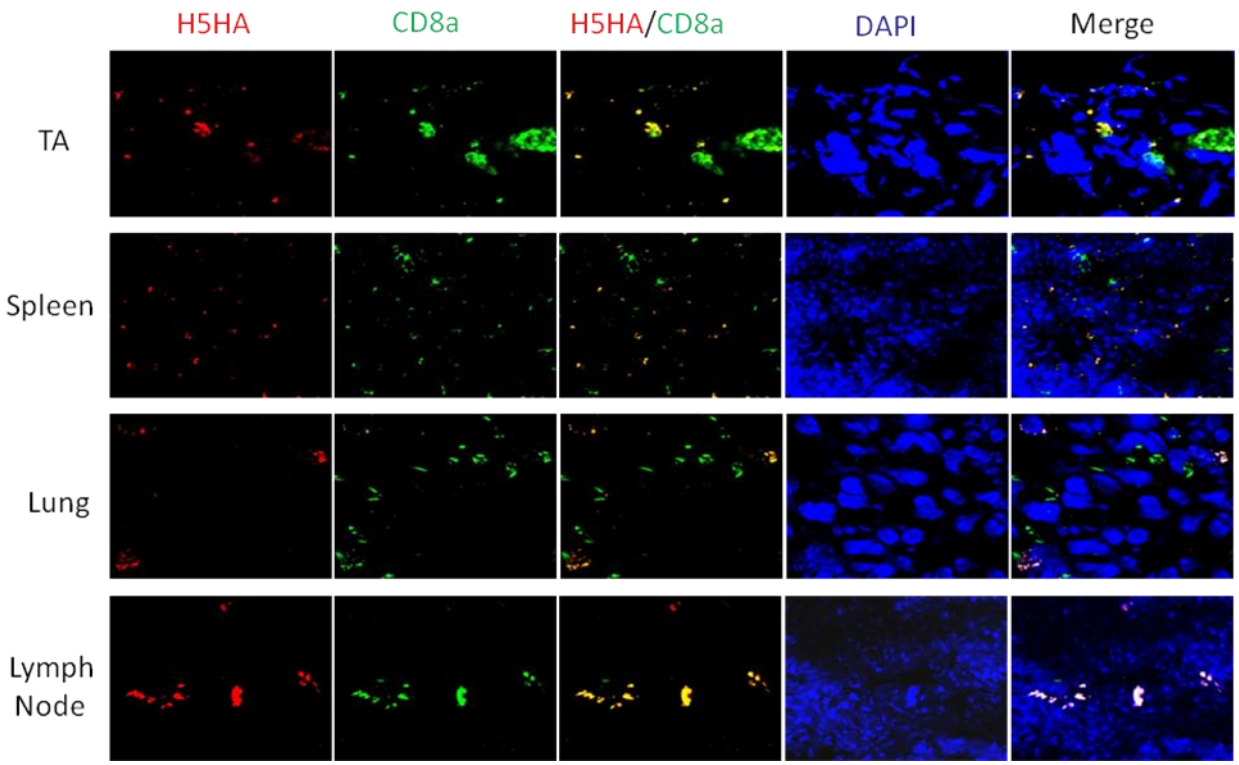
In support of the design of DNA plasmid encoding influenza protein H5N1 HA, the DNA sequence coding therapeutic gene H5HA has been successfully constructed into the pVAX1 backbone. In order to develop effective vaccine formulations, the goal of this experiment is to determine what cell populations accumulate the DNA and express the transgene after administration of the pVAX1-H5HA plasmid DNA in the skeletal muscle in a mouse.

10 µg of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in Balb/c mice (5 mice/group). The animals were sacrificed on day 7 after i.m. injection. Tibialis anterior muscle, lymph node, spleen and lung were harvested, and embedded in Tissue-Tek OCT, rapidly cooled and stored at –80°C for performing immunohistochemistry. Double staining immunofluorescence was performed in frozen tissue sections cut on plain glass slides. The glass slides were sequentially treating with a) monoclonal mouse anti-His-tag antibody (Cellsignaling, Danvers, MA) to detect His-tagged H5HA antigen, cell-specific Abs for macrophages (CD11b) (eBioscience, San Diego, CA), cytotoxic T cells (CD8a) (BioLegend, San Diego, CA) and T-helper cells (CD4) (Abcam,

Cambridge, MA). Specifically, 12 µm frozen sections of tissues (four slices per tissue specimen) were fixed in ice cold methanol and then blocked for 10 mins in 5% BSA inTris-buffered saline at room temperature, rinsed two times and incubated overnight at 4ºC with anti-His-tag and cell marker primary antibodies. After that the sections were rinsed three times with PBS and incubated 1 h at room temperature with secondary anti-species (rat/mouse) antibodies conjugated to a Alexa Fluor 594 (red)/Alexa Fluor 488 (green) (Invirtogen, Carlsbad, CA) were used. Two negative control specimens (PBS injected mice) were also used in this protocol. Finally, the samples were counterstained with DAPI and analyzed by Zeiss 410 Confocal Laser Scanning Microscope equipped with an Argon-Krypton Laser 595 nm (H5HA expression), 488 nm (cell marker) and 647 nm (nucleus) to visualize the co-localization (yellow) of HA (red) and cell-specific marker (green). The images were digitally superimposed to determine which cells express the transgene.

In the muscle tissues the H5HA gene was expressed in macrophages and T-cells after the administration of naked DNA or DNA/P85 or SP1017 in tibialis anterior muscles (Figure 8, 9 and 10). H5HA gene was also expressed in cytotoxic T-cells and T-helper cells in the muscles as well as in lungs, spleens and draining lymph nodes (Figure 8, 9 and 10). Figure 11 presents the negative control specimens from PBS injected mice. Here we report that *H5HA gene expressed, co-localized in Antigen Presenting Cells (APC) such as macrophages and translocated to the distal sites*. These results demonstrated that in response to administration of DNA and DNA/P85 or SP1017 the APC incorporated, expressed and transported the H5HA transgene to the central immune organs.




H5HA
CD8a
H5HA/CD8a
DAPI
Merge
TA
Spleen
Lung
Lymph Node

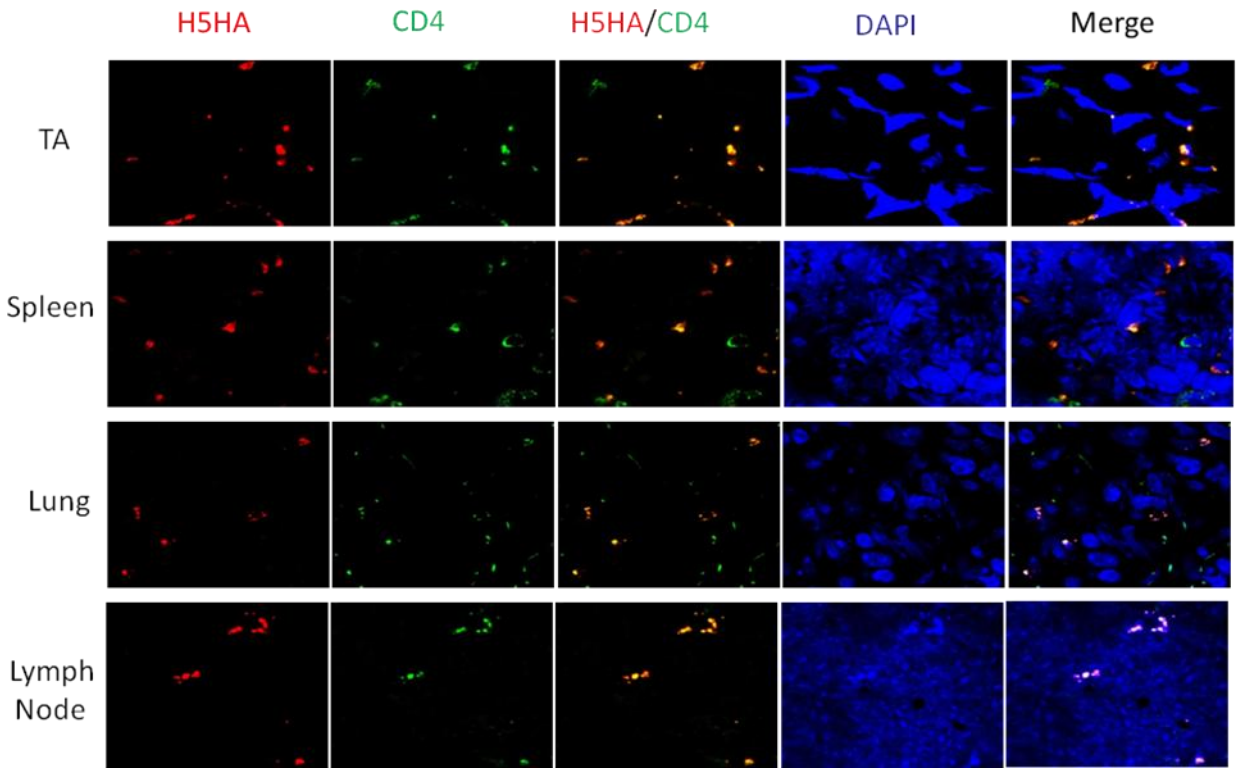
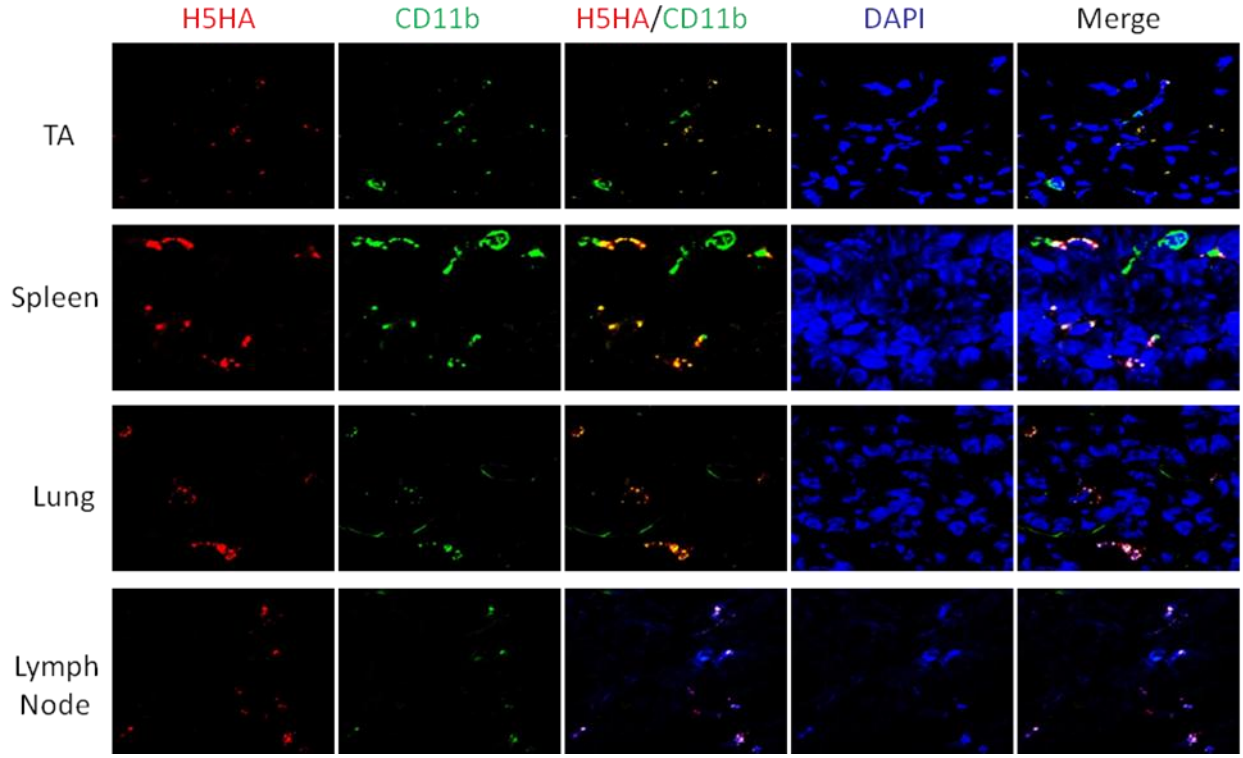

H5HA
CD4
H5HA/CD4
DAPI
Merge
TA
Spleen
Lung
Lymph Node

Figure 8. Confocal imaging of H5HA expression in macrophages (CD11b) and T-cells (CD8a and CD4) in muscle (Tibialis Anterior, TA), spleen, lung and draining lymph nodes after single *i.m.* injection of naked pH5HA DNA. The color staining corresponds to macrophages/T-cells (green), nucleus (blue) and H5HA (red). The 3rd and last panels in each row digitally superimposed to visualize the colocalization (yellow or white) Magnification 63x with 2x zoom.



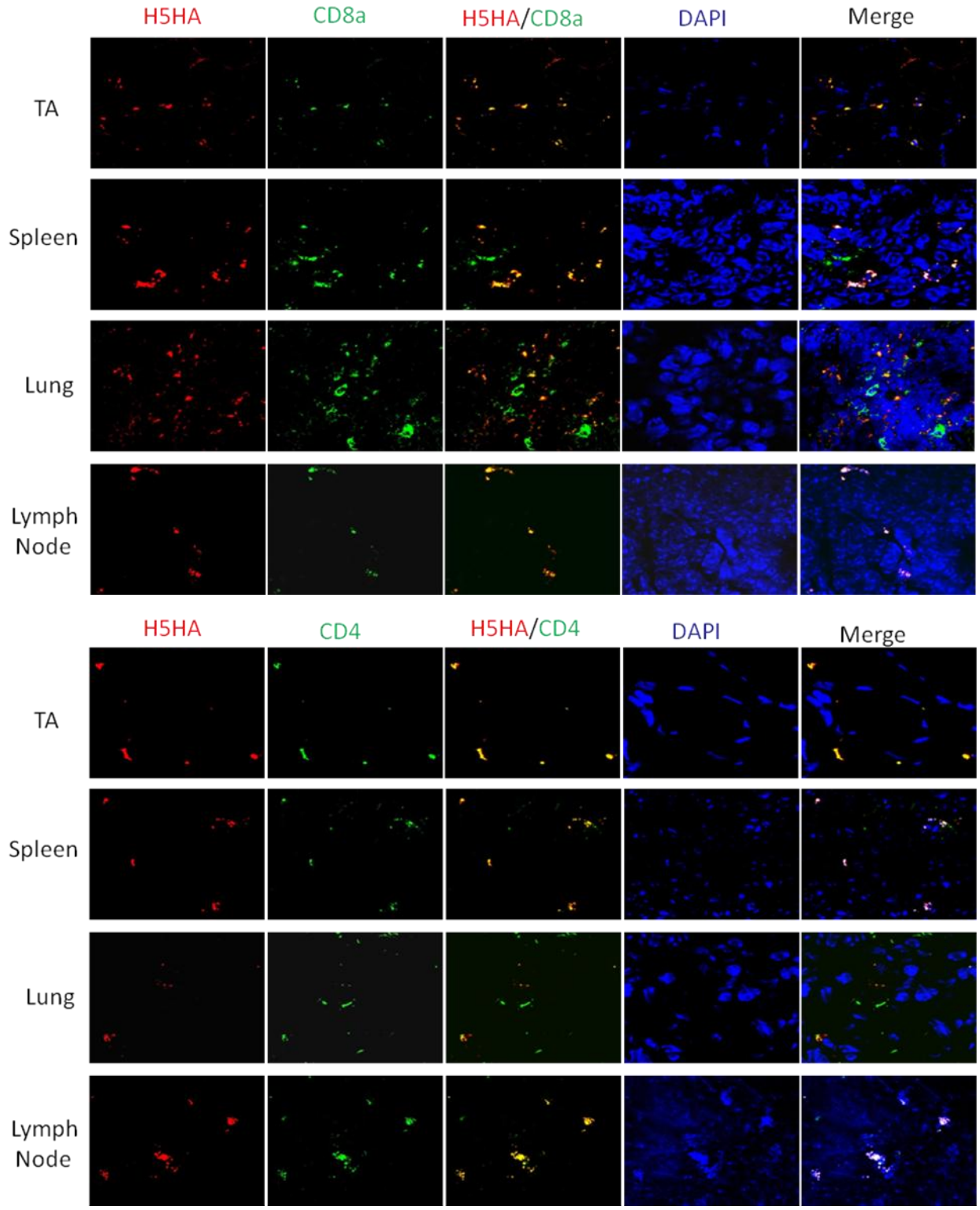
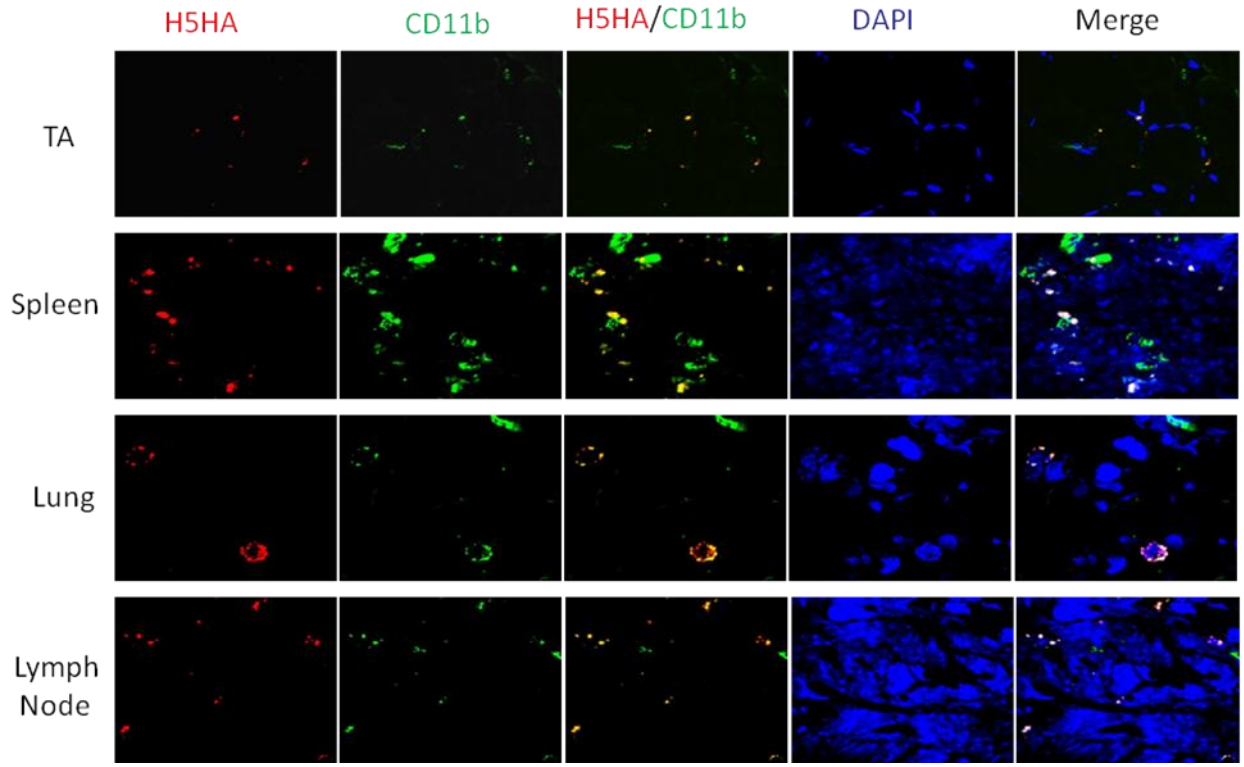


Figure 9. Confocal imaging of H5HA expression in macrophages (CD11b) and T-cells (CD8a and CD4) in muscle(Tibialis Anterior, TA), spleen, lung and draining lymph nodes after single

i.m. injection of pH5HA +0.3% P85. The color staining corresponds to macrophages/T-cells (green), nucleus (blue) and H5HA (red). The 3rd and last panels in each row digitally superimposed to visualize the colocalization (yellow or white) Magnification 63x with 2x zoom.



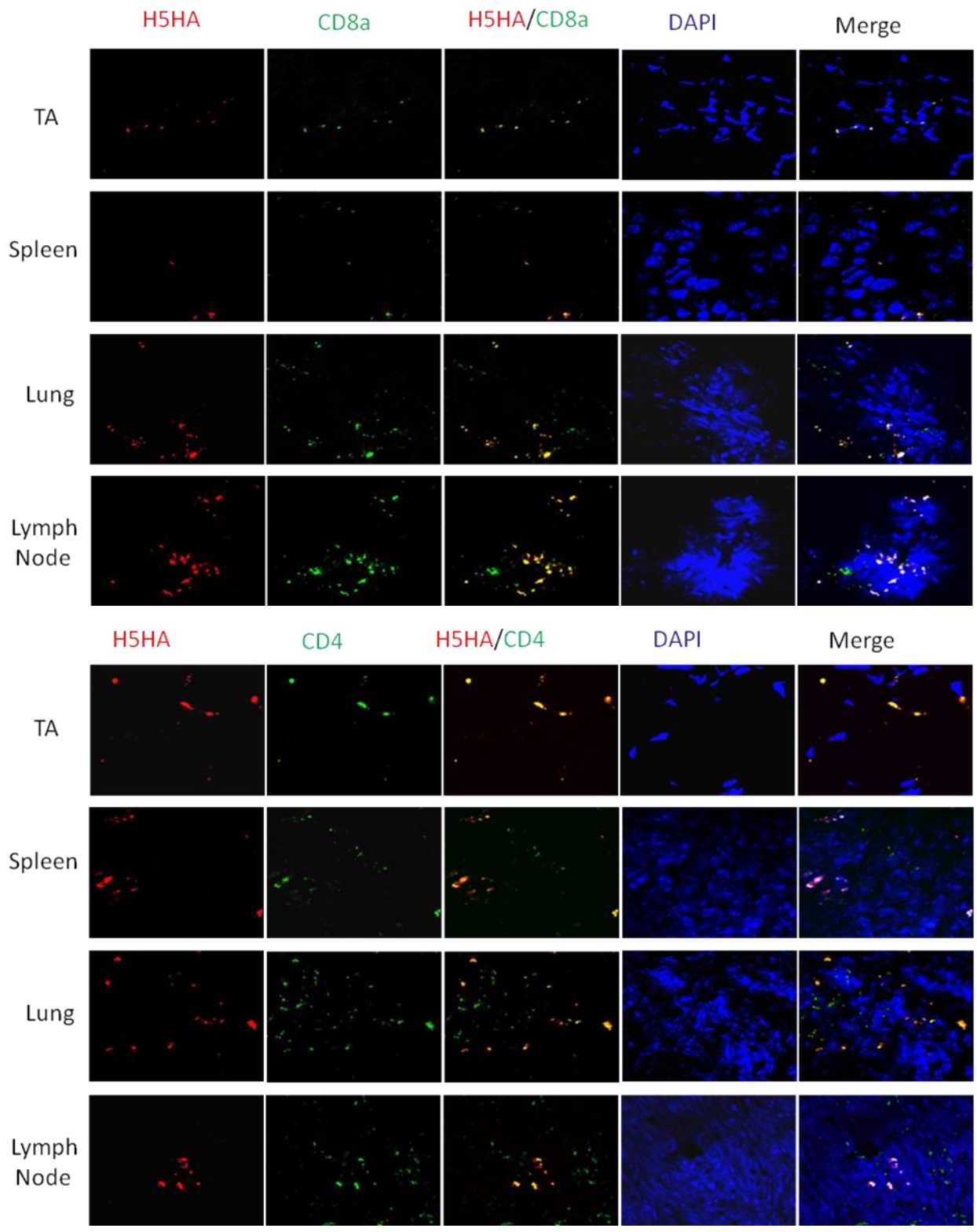
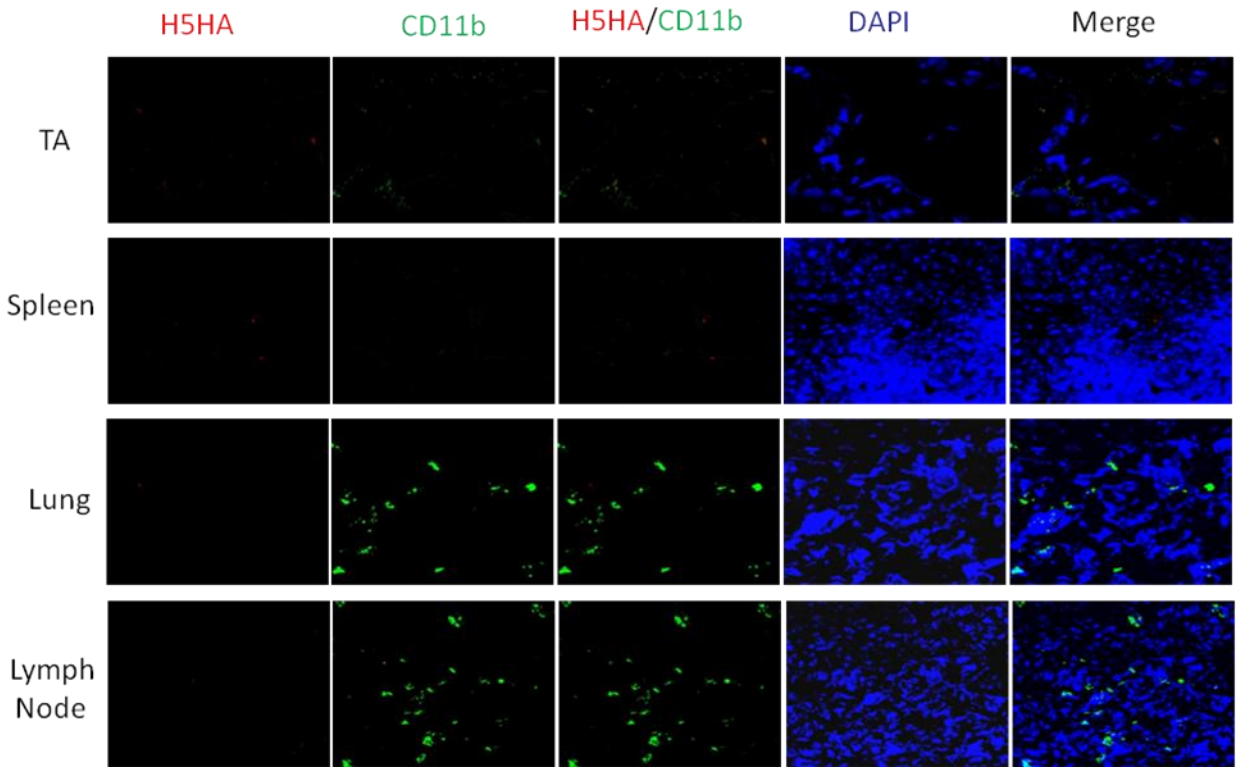


Figure 10. Confocal imaging of H5HA expression in macrophages (CD11b) and T-cells (CD8a and CD4) in muscle (Tibialis Anterior, TA), spleen, lung and draining lymph nodes after single

i.m. injection of pH5HA +0.01% SP1017. The color staining corresponds to macrophages/T-cells (green), nucleus (blue) and H5HA (red). The 3rd and last panels in each row digitally superimposed to visualize the colocalization (yellow or white) Magnification 63x with 2x zoom.



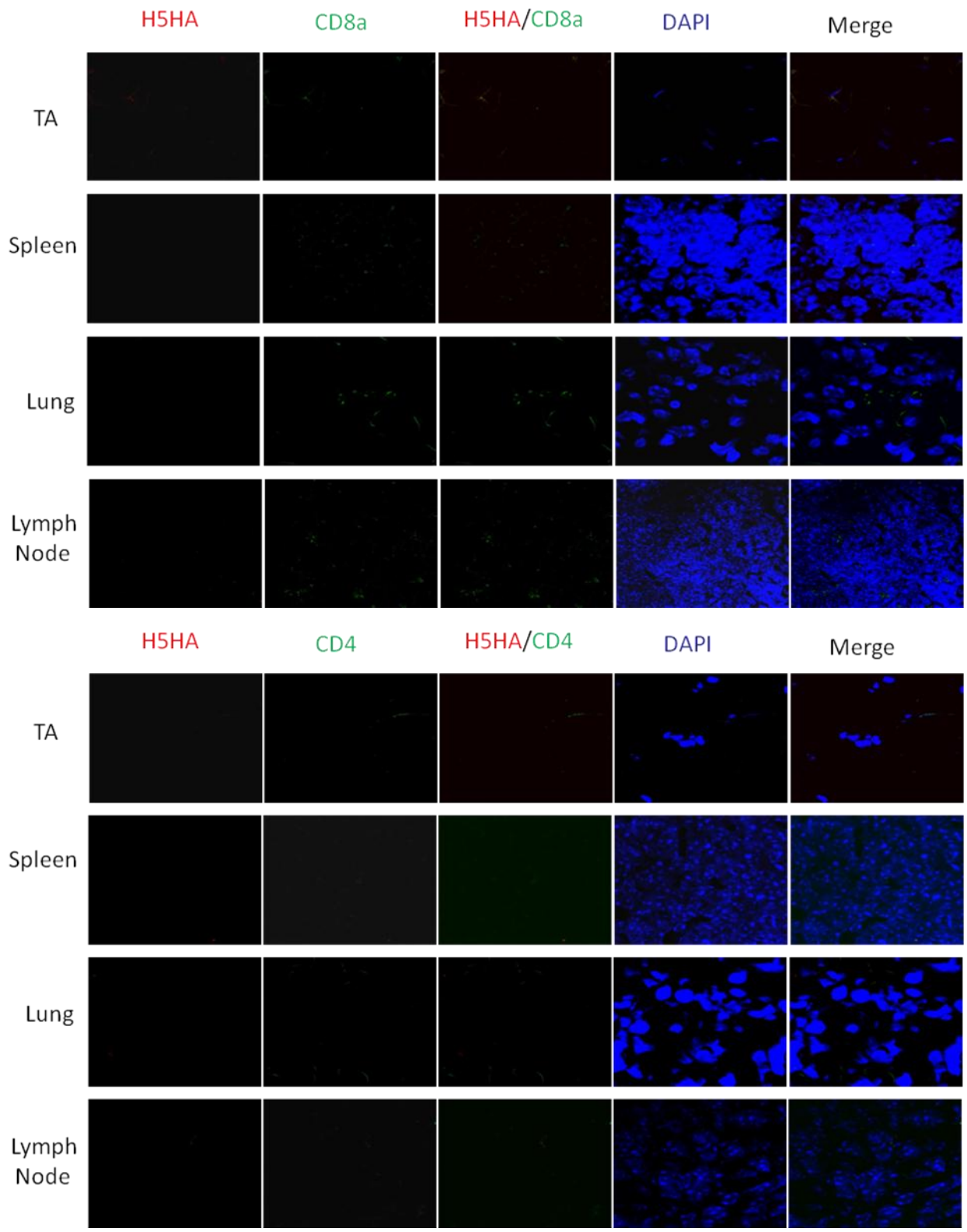
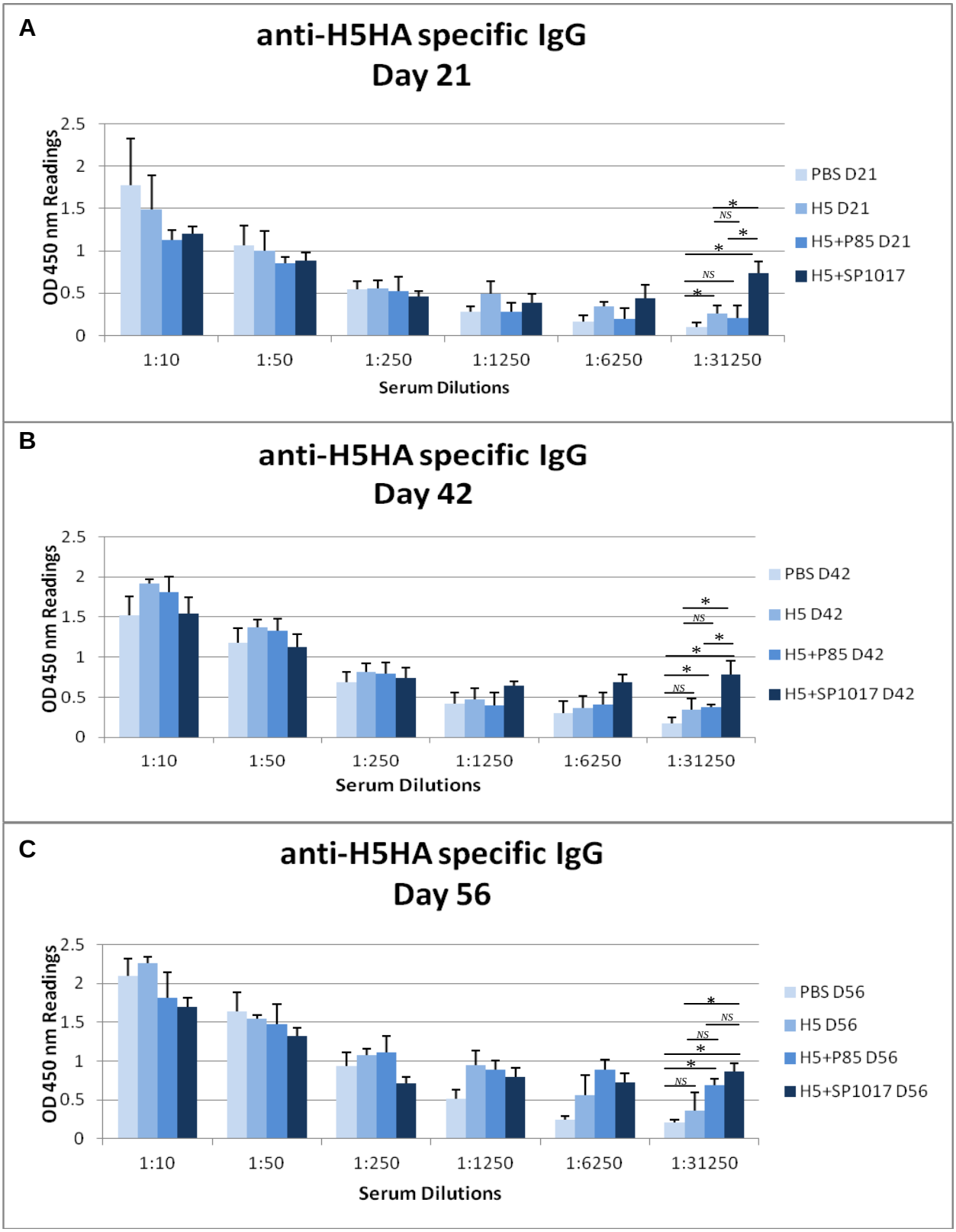


Figure 11. Confocal imaging of H5HA expression in macrophages (CD11b) and T-cells (CD8a and CD4) in muscle (Tibialis Anterior, TA), spleen, lung and draining lymph nodes after single

i.m. injection of PBS. The color staining corresponds to macrophages/T-cells (green), nucleus (blue) and H5HA (red). The 3rd and last panels in each row digitally superimposed to visualize the colocalization (yellow or white) Magnification 63x with 2x zoom.

In order to develop effective vaccine formulations, the goal of this experiment is to determine whether immunization with Pluronic/pVAX1-H5HA DNA vaccine can produce an immune response in a mouse. 50 µg of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. *in tibialis* anterior muscles in Balb/c mice (4 mice/group) and boosted on D29 with 50 µg of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017. Blood sera from individual mice were collected on day 21 (D21), 42 (D42) and 56 (D56). H5HA specific antibodies in blood sera were detected by enzyme-linked immunosorbent assay (ELISA). H5HA protein was purified from HEK-293T cells transfected with pH5HA using MagneHis Protein Purification System according to manufacturer's protocol (Promega, Madison, WI). 96-well microtiter plates (Nunc Life Technollogies, Rochester, NY) were coated with 5 µg/mL purified H5HA in PBS overnight at 4ºC. Plates were blocked for 3 h at room temperature with 1 % BSA/ PBS. 100 µL of serum dilutions were added to the plates and incubated for overnight at 4ºC. Plates were washed 3 times, and HRP conjugated mouse IgG detection antibody (Sigma, St. Louis,MO) diluted 1:3,000 was added to wells followed by 2 h incubation at room temperature. TMB peroxidase substrate (Bethyl Laboratories,Inc. Montgomery,TX) then added to the wells and allowed to develop for 30 min at room temperature. Optical density measurements were done at 450 nm.

In order to determine if our Pluronic/pVAX1-H5HA DNA vaccine formulations induce antibody responses we performed the ELISA and our data shown in Figure 12. The present study demonstrated that pH5HA DNA vaccine with/without Pluronics could induce specific IgG antibodies in mice (Figure 12). All three DNA-based vaccine formulations were immunogenic and induced booster responses. However, the IgG antibody was detectable on the day 21 in the mice vaccinated with pH5HA DNA formulated with SP1017 and maintained the same high level until day 56 (Figure 12 A, B, C and F). There was no booster response observed in this group. Whereas, in the mice groups immunized with naked pH5HA DNA or pH5HA DNA with 0.3% w/v P85, a significant increase of specific IgG antibody levels were observed in blood serum on day 56 (Figure 12 A, B, C, D and E). These data indicated that Pluronic/pVAX1-H5HA DNA vaccine formulations could induce a primary antibody response and that the level of the induced total IgG antibody was long lasting.


A
anti-H5HA specific IgG
Day 21
OD 450 nm Readings
2.5
2
1.5
1
0.5
0
1:10
1:50
1:250
1:1250
1:6250
1:31250
Serum Dilutions
PBS D21
H5 D21
H5+P85 D21
H5+SP1017
NS
*
B
anti-H5HA specific IgG
Day 42
OD 450 nm Readings
Serum Dilutions
PBS D42
H5 D42
H5+P85 D42
H5+SP1017 D42
C
anti-H5HA specific IgG
Day 56
OD 450 nm Readings
Serum Dilutions
PBS D56
H5 D56
H5+P85 D56
H5+SP1017 D56

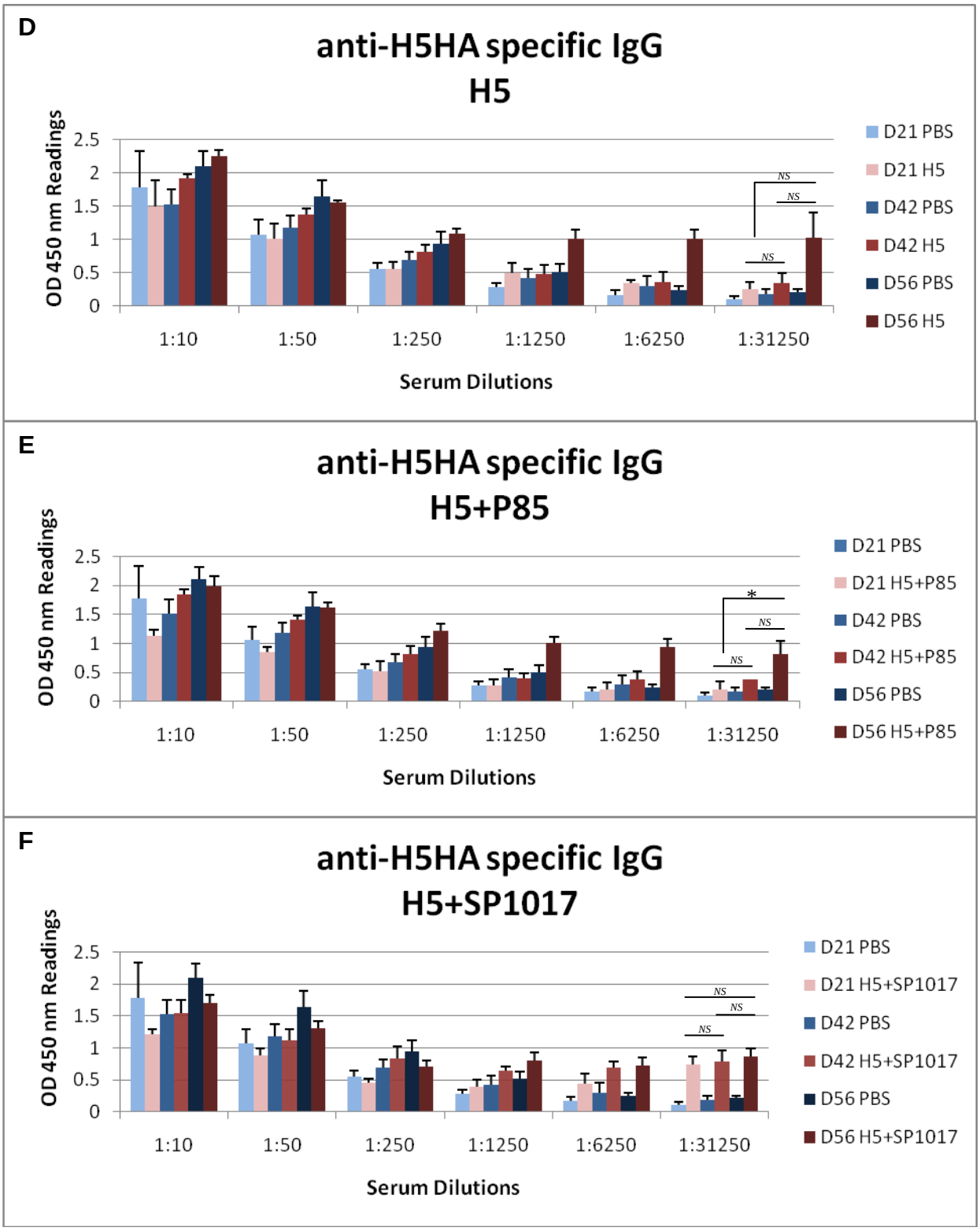

D
anti-H5HA specific IgG
H5
OD 450 nm Readings
2.5
2
1.5
1
0.5
0
1:10
1:50
1:250
1:1250
1:6250
1:31250
Serum Dilutions
NS
NS
NS
D21 PBS
D21 H5
D42 PBS
D42 H5
D56 PBS
D56 H5
E
anti-H5HA specific IgG
H5+P85
OD 450 nm Readings
2.5
2
1.5
1
0.5
0
1:10
1:50
1:250
1:1250
1:6250
1:31250
Serum Dilutions
*
NS
NS
D21 PBS
D21 H5+P85
D42 PBS
D42 H5+P85
D56 PBS
D56 H5+P85
F
anti-H5HA specific IgG
H5+SP1017
OD 450 nm Readings
2.5
2
1.5
1
0.5
0
1:10
1:50
1:250
1:1250
1:6250
1:31250
Serum Dilutions
NS
NS
NS
D21 PBS
D21 H5+SP1017
D42 PBS
D42 H5+SP1017
D56 PBS
D56 H5+SP1017

Figure 12. **Total serum IgG antibody responses to H5HA.** 50 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µL of PBS were administered in tibialis anterior muscles (4 mice/group) and boosted with the same formulations on D29. Sera from individual mice were taken 21, 42 and 56 days after administration of vaccine formulations and were tested for binding to H5 hemagglutinin by ELISA. Panel A, B and C are the H5HA specific IgG antibody titers by ELISA in sera at days 21, 42 and 56 (D21, D42 and D56) and panel D, E and F are the titers of H5HA specific IgG antibodies among different forms of vaccine. All data are presented as means ± standard deviations and analyzed with Student's *t*-test (n=4). **P*<0.05 and *NS* is not significant at 0.05 levels.

To achieve a milestone related to optimization of antibody response generated by DNA vaccine, we incorporated the boosting step into vaccination regimen. As was demonstrated in Figure 12, the boosting resulted in prolongation of antibody response generated by free HA DNA and its formulation with Pluronic P85. However, it is important to note that the boosting practically did not affect the level of antibody production after vaccination with HA DNA/SP1017 formulation. It was significantly higher on days 21 and 42 compared to other vaccine formulations studied. As a next step, we evaluated the efficacy of the identified vaccine formulations administered using boosting regimen. The goal of these experiments was to determine whether immunization with Pluronic/pVAX1-H5HA DNA vaccine can protect the mice against Low Pathogenicity H5N1 viral challenges. For these initial experiments on testing the protective efficacy of the vaccines mice viral challenge was administered via intranasal application. Dose of virus administered to the mice will be more accurately controlled using this methodology.

Vaccination of the animals was performed as follows: 50 µg of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in Balb/c mice (5 mice/group) and boosted on day 29 (D29) with 50 µg of pVAX1-H5HA plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017. On day 63 after first vaccination the animals were challenged via intranasal administration of 30 µl of neat $1.9x10^6$ $TCID_{50}$ Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). The virus inoculums were obtained from Benchmark Biotech. The infected mice were observed daily for signs of illness or discomfort, and animal weight was measured for 14 days. All mice in the control group (PBS treatment) survived the challenges while exhibiting higher weight loss and delayed recovery compared to the vaccinated groups (Figure 13 A and B). One of the mice from H5+SP1017 group suddenly died on day 4 post-challenge and another mouse from H5 group lost 30% of its original weight and had to be euthanized on day 6 (Figure 13 B). All animals in H5+P85 vaccinated group survived the challenge study, and exhibited a weight loss of only 15% of their average pre-challenge body weight on day 7 and regained their original body weight (Figure 13 A and B) on day 14. At the end point of the experiment mice were euthanized.

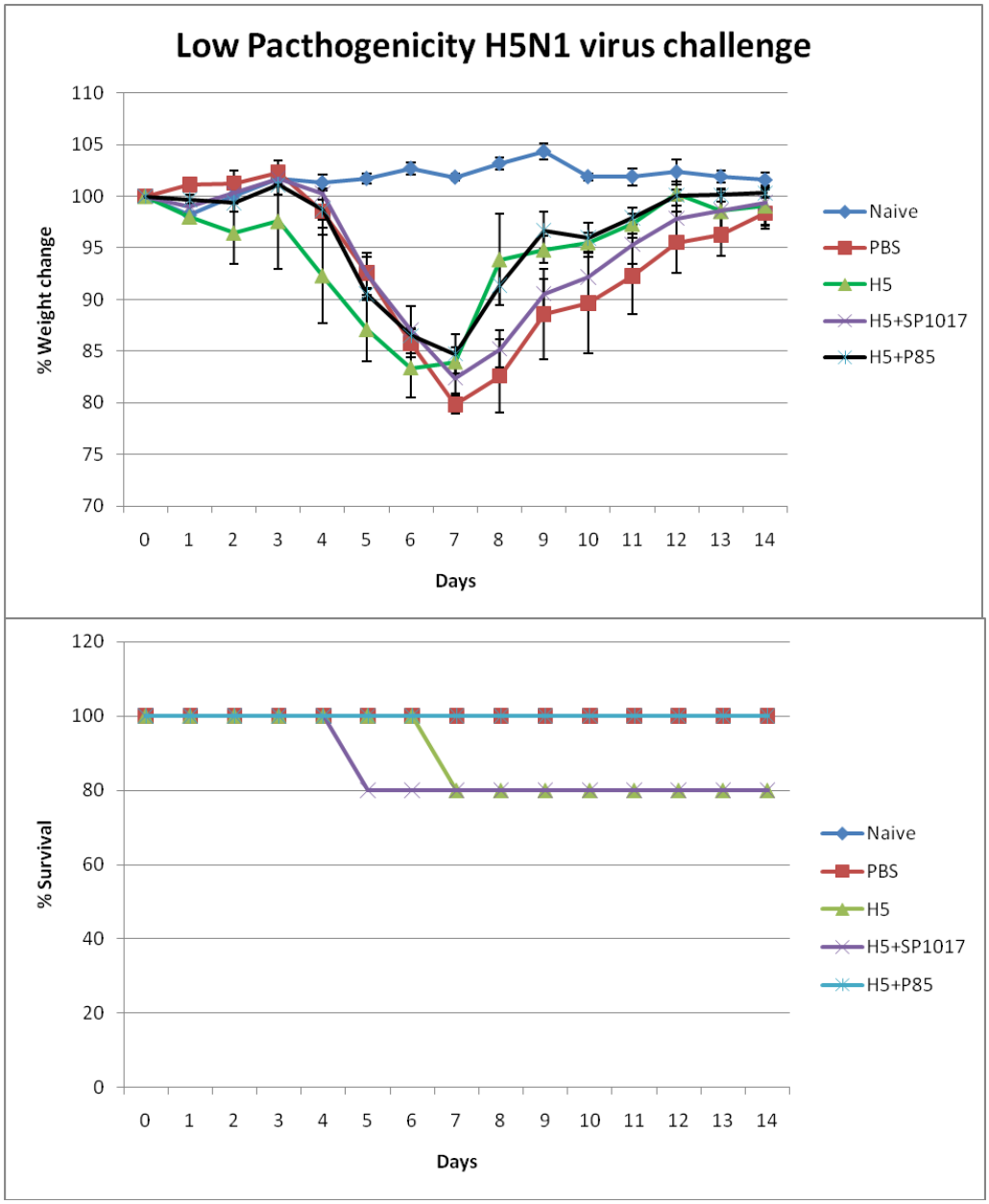


Figure 13. 50 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µL of PBS were administered in tibialis anterior muscles of BALB/C mice (5 mice/group) and boosted with the same formulations on D29. Mice were challenged intranasally on D63 with 30 µl of neat $1.9x10^6$ $TCID_{50}$ Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). (A) body weight changes (data expressed as a percentage of the pre-challenged body weight and presented as mean ± SEM, *n* =3-5) and (B) animal survival was daily monitored for 14 days (Day 0 = day virus challenged).

To determine the minimum amount of virus that would give a 100% mouse infectious dose (MID_{100}) of the low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG), dilutions of neat $1.9x10^6$ $TCID_{50}$ of 1:10 and 1:100 were made in sterile PBS. PBS and undiluted virus were used as controls. Either 30 or 60 microliters were instilled in the nares of

Balb/c mice and weight loss/gain was observed for 13 days. Three (3) mice were used per treatment group and two (2) mice were used in the control (PBS) group. All mice, excluding both sets of PBS controls and the Naive mice showed marked weight loss by day 8, but all mice recovered and body weight was increasing to near starting weight by day 13. It was determined that further work was necessary and that viral dilutions between 1:10 and 1:100 in PBS should be done to further define the MID $_{100}$ of the virus stock (Figure 14).

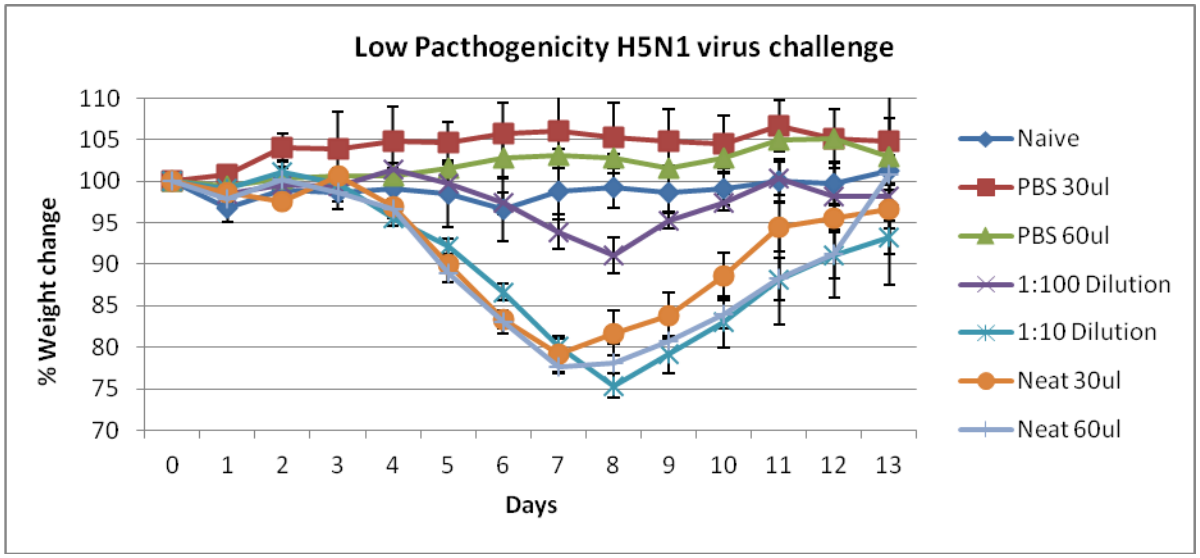


Figure 14. Mice were challenged intranasally with 30 µl of neat 1.9x10^6 $TCID_{50}$ and diluted Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). Body weight changes (data expressed as a percentage of the pre-challenged body weight and presented as mean ± SEM, *n* =2-3) and monitored for 13 days (Day 0=day virus challenged).

To better determine the 100 % mouse infectious dose (MID_{100}) of the 1.9x10^6 $TCID_{50}$ low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG), dilutions of 1:10, 1:20, 1:30, 1:40, and 1:50 were made in PBS. PBS and undiluted virus were used as controls. 30 microliters were instilled in the nares of Balb/c mice and weight loss/gain was observed for 13 days. Three (3) mice were used per treatment group and two (2) mice were used in the control (PBS) group. All mice, excluding the PBS controls showed marked weight loss by day 8, but all mice recovered and body weight was increasing to near starting weight by day 13. The Influenza standard for infection is weight loss below 80% of the original body weight. *It was determined that a 1:10 dilution in PBS of the virus stock was the minimum MID_{100} as this was the only dilution that achieved 100% of mice with weight loss below 80%; this dilution will be used for further experiments* (Figure 15 A). The 1:10 virus challenged mice and the PBS control group from these experiments (Figure 15 A), were re-challenged with a 1:10 virus dilution (Figure 15 B) and observed for 14 days. All animals in this re-challenged group survived the challenge study, and exhibited no change of body weight as PBS controlled group (Figure 15 B). This result demonstrated that mice challenged with 1:10 dilution of 1.9x10^6 $TCID_{50}$ low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG) protected the mice against the influenza.

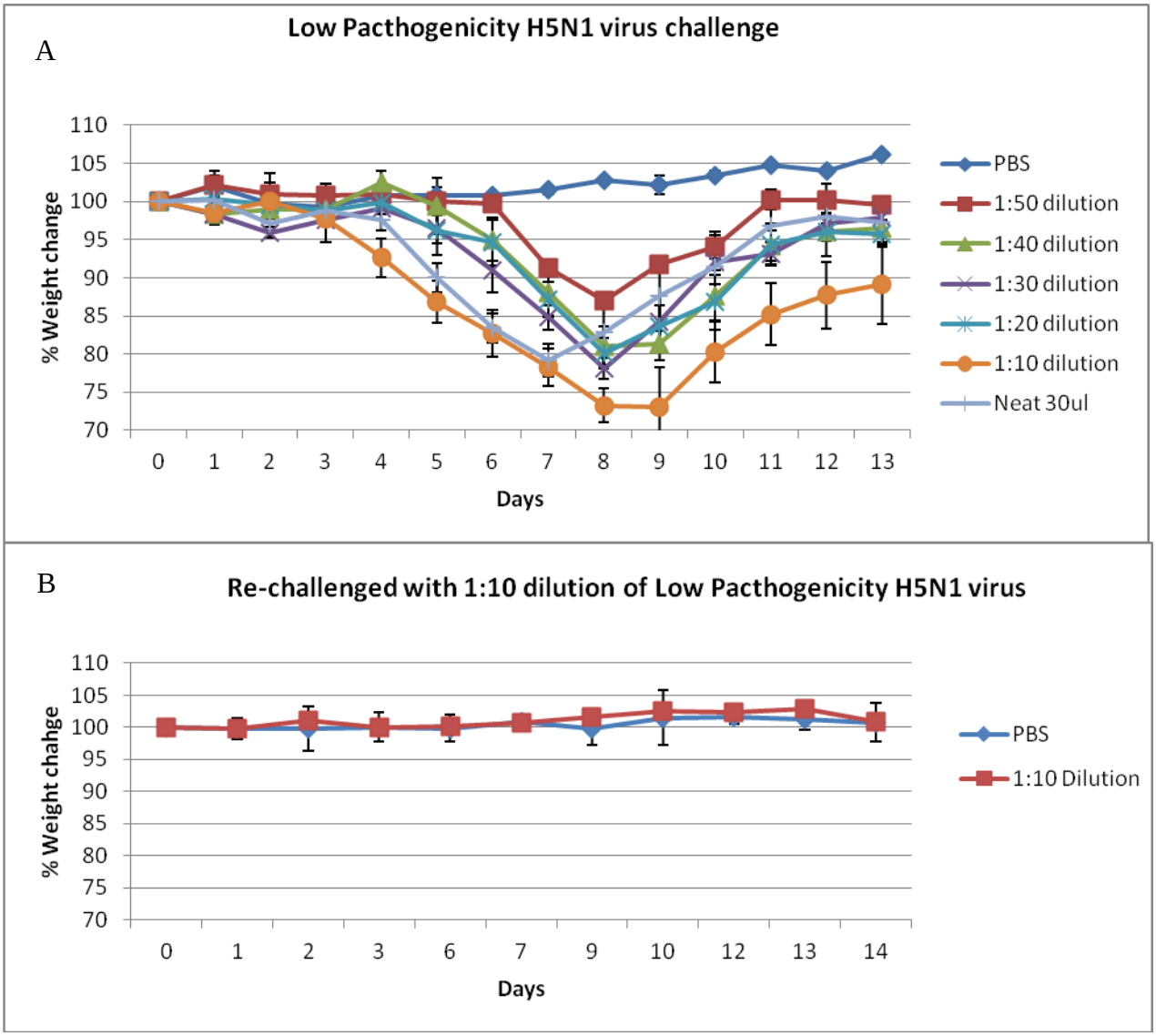


Figure 15. A. Mice were challenged intranasally with 30 µl of neat $1.9x10^6$ $TCID_{50}$ and diluted Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). Body weight changes (data expressed as a percentage of the pre-challenged body weight and presented as mean ± SEM, *n* =2-3) and monitored for 13 days (Day 0=day virus challenged). B. PBS and 1:10 dilution group of mice were re-challenged with the same formulations on day 28 and body weight changes were monitored for 14 day.

Currently, we are in the process of determining the levels of virus in the blood, lungs and spleens of the viral-challenged mice with 1:10 dilution of $1.9x10^6$ $TCID_{50}$ low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG) over the course of infection from day 2 through day 12. We will also challenge the pH5HA with/without Pluronics vaccinated mice with the same virus dilution to determine the efficacy of our vaccine formulations.

We also continued studies in the task aimed to maximize levels and duration of transgene expression after administration in skeletal muscle in a mouse. Previous studies with Pluronic P85 mediated *in vivo* gene delivery have reported increase in reporter protein expression up to an order of magnitude when P85 is co-delivered with plasmid DNA (pDNA) compared to pDNA alone in healthy Balb/c mice [1]. This increase in gene expression was also found in distal lymphoid organs (draining lymph nodes and spleen) in addition to injected muscle. Our rationale here was to study the effect of role of immune cells in Pluronic mediated increase in transgene expression. We performed Pluronic formulated pDNA injections in bilateral tibialis anterior muscles preinjected with increasing concentrations of P85 i.e. local inflammation model to study the role played by immune cells in increasing the transgene expression by increasing the local inflammation (Figure 16).

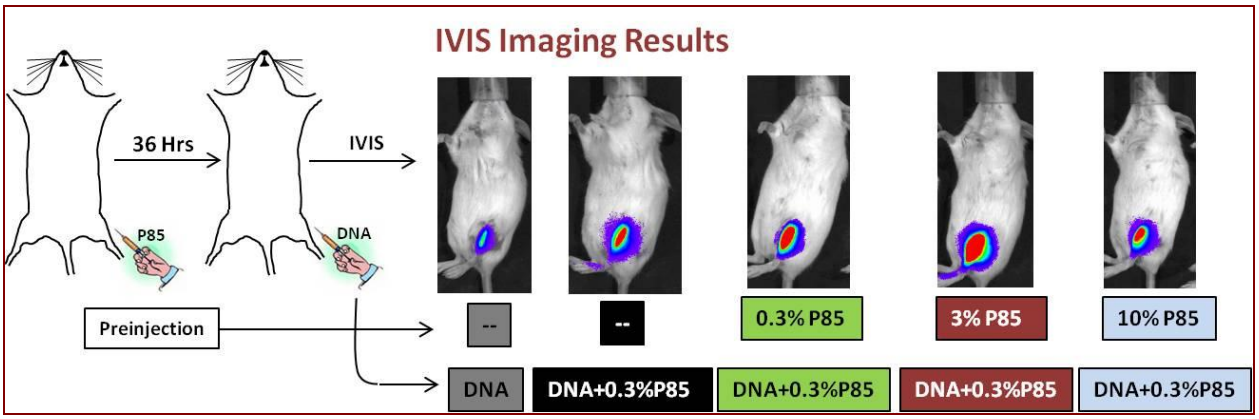


Figure 16. **Scheme of experiment and representative image of non-invasive *in vivo* imaging (Xenogen IVIS 300) results.** Animals were preinjected with increasing concentrations of P85 and then injected DNA 36 hours later. (n=4**)**

To prove our hypothesis we induced local infiltration of immune cells in bilateral tibialis anterior muscle of balb/c mice by injecting increasing dose of P85 (0.3 %, 3% and 10%) in 50 ul of PBS (preinjection model) followed by 10ug DNA+P85 (0.3%) injections at 36 hour (i.e. estimated neutrophil infiltration peak) after pre-injection. DNA/DNA+P85 injections without preinjections were used as controls for preinjection model. Luciferase reporter protein expression was monitored by injecting D-luciferin (150 mg luciferin/kg body weight) *i.p.* followed by non invasive *in vivo* bioluminescent imaging (exposure for 5 seconds) using Xenogen IVIS 200 imaging system. Kinetics of protein expression was monitored till day 50 post DNA injection with a peak of luciferase expression at day 10 (Figure 17a). Quantification of images at D10 demonstrated that animals preinjected with 3% P85 expressed luciferase activity 13 and 7 times compared to DNA alone and DNA+P85 respectivey (Figure 17b). Infiltration of immune cells (e.g. neutrophils, macrophages etc) have long been corelated with repair and regeneration of muscle tissues [2]. Increase in gene expression with increasing P85 (0.03% and 3%) could be explained by increased immune cell infiltration and their role in increasing the transgene expression and tissue repair. On the contrary 10% P85 preinjection may induce tissue injury beyond repair which could be the reason for decreased gene expression.

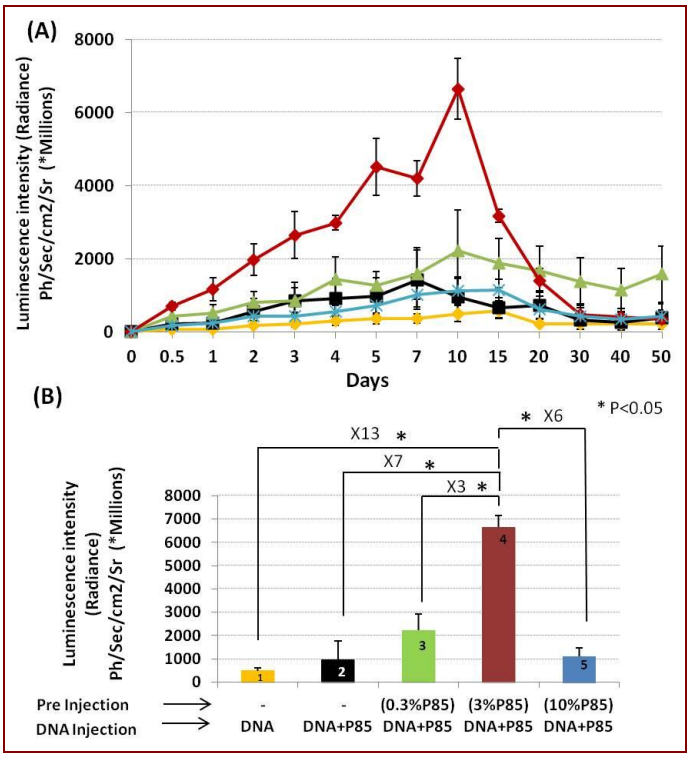


Figure 17. **Effect of P85 preinjections on gene expression** after single i.m. injection of 10 ug plasmid DNA in 50 mL PBS (A). Quantitative data of in vivo imaging study at day 10 (B). Data are mean SEM (n =4). P values were obtained by the means of Student's t test.

We further attempted to understand the role of P85 in increasing the transgene expression in immune cells by conducting *in vitro* studies. Toward this goal Balb/c macrophage cells RAW264.7 were coincubated with increasing concentrations of P85 and 1 ug of pDNA for 4 hours at $37^{O}C$, 5% CO_2, washed with PBS and media replaced. After 16 hours cells were washed and lysed with Promega lysis buffer (1X) and supernatant used for quantification of luciferase protein. The assay was performed as follows: 10 ul of the supernatant was added to luminometric tubes and supplemented with 100 ul of luciferase substrate solution (Promega, Madison, WI). Light emission was measured with a luminometer (Promega, Madison, WI) for a period of 20 s. Surprisingly, we found that 1% P85 when mixed with 1 ug plasmid DNA increased transgene levels upto 2000 times compared to DNA alone in RAW 264.7 macrophages *in vitro* (Figure 18). DNA alone and GenePORTER3000 transfection reagent were used as negative and positive controls respectively.

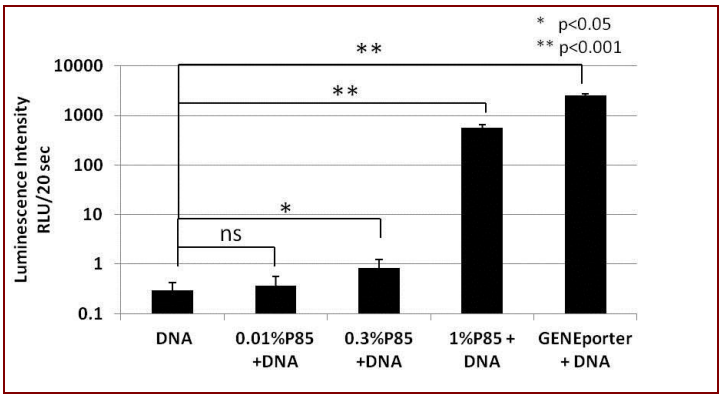


Figure 18. **Quantitative data of *in vitro* luciferase activity:** RAW264.7 macrophage cells were coincubated with 1ug gWIZluc and different concentrations of Pluronic P85. Cells were exposed to P85+DNA for 4 hours in serum free media and assay was performed after 16 hour of incubation. Data are mean±SEM (n=3) p values are obtained by means of student's t test.

Platform C

As demonstrated in prior studies mannosylated BICs presented no significant advantages over non-functionalized BICs as either the mouse bone marrow macrophages (BMM) expressed moderate level of mannose receptors or the transfection efficiency in human monocyte-derived macrophages was low (see previous report). Multiple recent publications have demonstrated the feasibility of folate mediated active targeting delivery of genes to macrophages [2-6], therefore, we applied Folate functionalized BICs as an alternative strategy.

Alternative strategy 1: Folate functionalized BICs (Folate-BICs)

Folate receptor levels in macrophage cells. To confirm the expression of folate receptors, we immunostained folate receptors in human monocyte-derived macrophage cells. As shown in Figure 19, folate receptor (green staining) is highly expressed in these human macrophage cells.

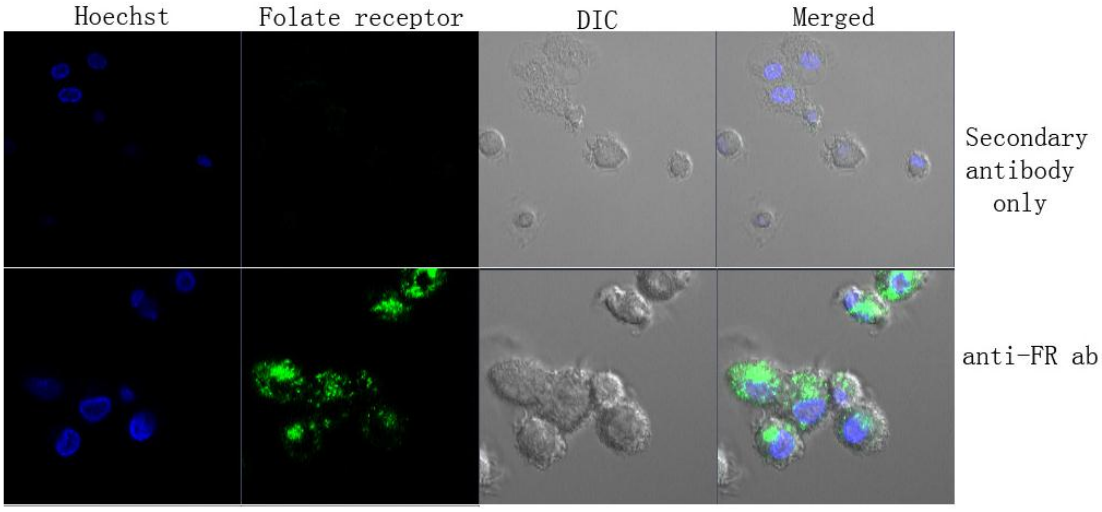


Figure 19. Folate receptor staining in human macrophage cells. Blue, nucleus; Green, folate receptor; Grey, DIC. Primary antibody: anti-FR rabbit polyclonal antibody; secondary antibody: Goat-anti-rabbit alexa 488 conjugated.

Folate-BICs. Folate-BICs were produced by mixing Folate functionalized cationic copolymers Folate-Poly(ethylene glycol)-b-Poly(l-lysine) (Folate-PEG-PLL) with opposite charged DNAs, the same approach to make mannose-BICs. BICs using PEG-PLL copolymer without folate targeting moiety (non-Folate-BICs) were used as a control. Cellular uptake of these BICs was determined by labeling DNA with YoYo-1. A clear trend (Figure 20) followed 0.5, 1 and 2 hours incubation times showing higher uptakes of Folate-BICs (Red bar) as compared to non-Folate-BICs (Blue bar), while concomitant addition of 1mM free folate was sufficient to compete out the uptakes of Folate-BICs (Black bar).

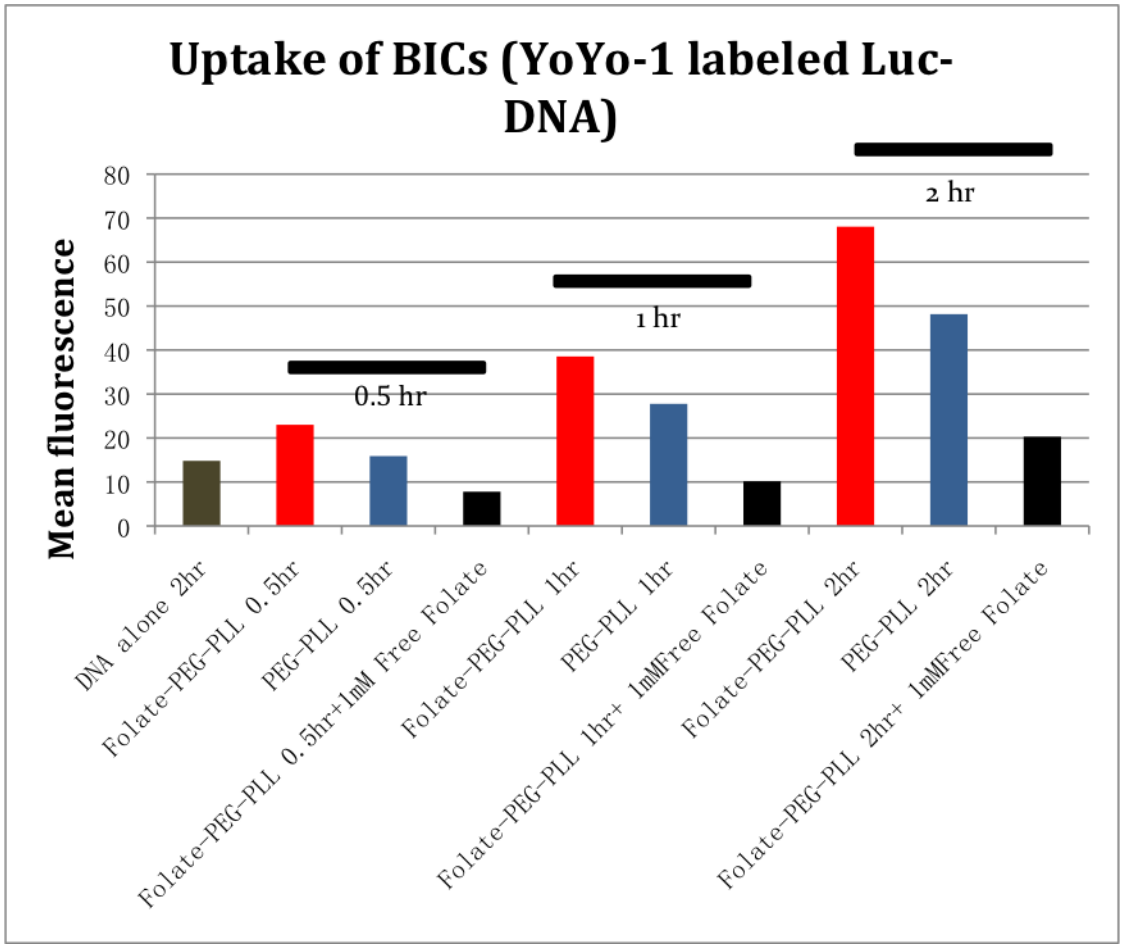


Figure 20. Uptake studies of YoYo-1 Labeled DNA in BIC complexes.

When transfecting RAW264.7 murine macrophage cells (Figure 21), Folate-BICs (Red bar) exhibited nearly 96 fold increase of luciferase expression over DNA alone (Brown bar), and approximately 1.5 times higher than the non-Folate BICs (50% increase) (Blue bar). In addition, co-incubate with free folate (Black bar) abolished the DNA transfection efficiency.

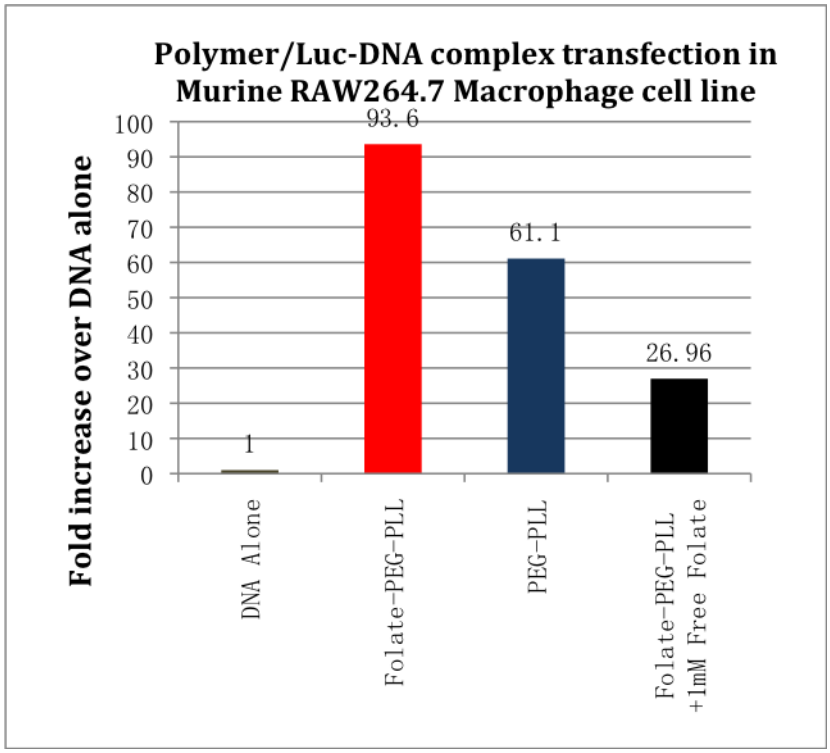
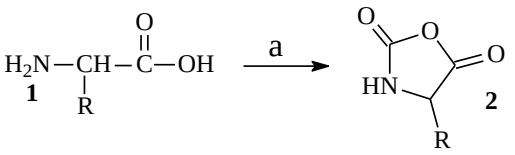


Figure 21. Transfection efficiency of different BICs in Murine Macrophage RAW264.7 cells.

Above results with merely 50% increase in cellular uptakes and gene expression for Folate-BICs over non-Folate-BICs prompted us to re-verify the polymer quality. Significant amount of impurities in the previously synthesized polymer products were identified possibly ascribing to degradation or unprecise purification, which demands the synthesis of a new batch high purity polymers. Proposed new synthetic pathway is described as following.

Methods and Schematics of the synthesis of Folate-Poly(Ethylene glycol)-Poly(L-Lysine) polymers (Folate-PEG-PLL$_{50}$) [7]

Synthesis of monomer (Lys-NCA) (Scheme 1): 5.0g (0.0178 mole) Nε-Carbobenzyloxy-L-Lysine (Lys(z)) was dissolved in 60 ml anhydrous THF and stirred to form suspension at room temperature (RT). The nitrogen was bubbled through the mixture during synthesis (about 3h). About 2.0g (0.00674mole) of triphosgene was dissolved in 15 ml of anhydrous THF and added to the above suspension drop by drops as injection to reaction solution. The mixture was heated up to 50^{o}C and left to proceed at reflux conditions. After the mixture become transparent, the solution was precipitate in anhydrous n-hexane (about 225 ml) and then stored at -20^{o}C overnight. Crude product formed during precipitation was recrystallized twice with anhydrous THF/Hexane mixture. The white solid (Lys-NCA) monomer was dried under vacuum for 24 h and characterized by ^{1}H-NMR ($CDCl_3$).

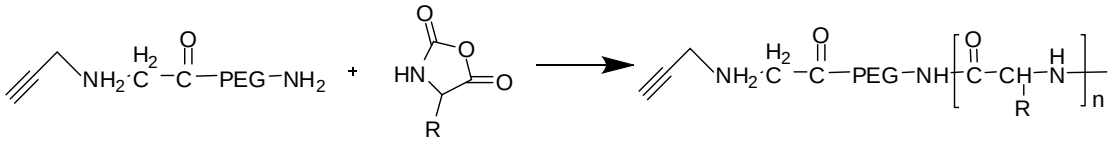


R: $(CH_2)_4NHCOOCH_2C_6H_5$

Scheme 1

Synthesis of Fmoc-PEG-Propargyl and NH_2-PEG-Propargyl: The 0.55g (100µmol, 1eq) Fmoc-PEG-NHS were dissolved in 3mL extra dry dichloromethane(DCM). Dilute 80µL (1200µmol, 12eq) propargylamine to 5mL DCM and mix with 3mL Fmoc-PEG-NHS solution. Mixture was stirred overnight at 4 ^oC and next day evaporate DCM and dissolve products in 15mL H_2O followed by dialysis against DD H_2O overnight with molecular weight cutoff 2000. 447.5mg white Fmoc-PEG-Propargyl products were obtained via lyophilization. Deprotection of Fmoc group was realized by 20% piperdine/DMF and for 2 h at RT, then the solvent were removed by rotary evaporation. Crude products were dissolved in DCM, precipitated with diethyl ether, and filtrated/dried under vacuum.

Synthesis of Propargyl-PEG-PLL(Z) (polymerization): The polymerization of propargyl-PEG-PLL (Poly-L-Lysine) were completed (Scheme 2, n=50). Briefly, 0.2g of NH2-PEG-Propargyl was dissolved in 5ml of anhydrous DMF and 3µl of triethylamine (TEA) was added to this solution upon stirring to convert NH3+ to NH2. Mixture was stirred continuously with nitrogen bubbling through the solution and temperature was maintained at about 35-40^oC. Calculated amount of Lys-NCA (612mg) which is equivalent to 50 lysine units per PEG chain was dissolved in 2ml of anhydrous DMF and injected dropwise to the reaction mixture. The reaction ampule was then argon bubbled, sealed and kept with stirring for 2 days. Reaction products was precipitated using excess of diethyl ether. The sample was filtered and the solid precipitate was re-dissolved in 5 ml DMF and precipitated with excess amount of ether. The precipitation procedure was repeated 3 times and solid polymer products were dried upon vacuum for 24 h followed by 1H-NMR analysis. Removal of Z protecting group was realized in trifluoroacetic acid(TFA) using a 33% hydrobromic acid solution in galatic acetic acid. The reaction mixture was drop-wise added to excess of cold diethyl ether to precipitate deprotected polymers. The precipitates were redissolved in methanol and precipted with diethyl ether twice.



R= $(CH2)_4$-NH-$COOCH_2Ph$

Scheme 2

Synthesis of 2-Azidoethylfolate: 443.42 mg folic acid (FA, 1 mmol) was dissolved in 20 mL anhydrous DMSO. 250.40 mg N,N'-dicyclohexylcarbodiimide(DCC, 1.2 mmol) and 13.04 mg 4-dimethylaminopyridine(DMAP, 0.1 mmol) were dissolved in 3 mL anhydrous DMSO and added to the FA solution under stirring at 40-41°C for 15 minutes. The activated FA was added dropwise to a stirring solution of 347.5 mg 2-Chloroethylamine hydrochloride

(3 mmol) in 10 mL anhydrous DMSO. Afterwards 0.2086 mL TEA(1.5 mmol) was added and the reaction mixture was stirred over night at room temperature in the dark. The urea byproduct was removed by filtration through glass wool and the approx. 30 mL solution was diluted with 30 mL water and 1 mL 1N NaOH, and precipitated with 1 mL 6 M HCl and dried under reduced pressure(0.140 mbar) over night. The solid was redissolved in 10 mL anhydrous DMSO and 195.20 mg NaN_3(3 mmol) were added and stirred for 3 h at 49-53 °C. The product was precipitated in a large excess of diethyl ether (1:20) and wash 3 times with approximately 20 mL ether and dried yellow solid was obtained.

Till current, we synthesized polymer propargyl-PEG-PLL (Poly-L-Lysine) and azido-folate. The final click reaction(Azide-Alkyne Huisgen Cycloaddition) to attach targeting group folate to propargyl-PEG-PLL polymer chain is in process.

Alternative strategy: Liposome/DNA complex (Lipoplex) formulations

A commercial *in vitro* transfection reagent GENEPORTERS demonstrated steady high efficiency for DNA transfection to murine macrophage cell line RAW264.7. However, this *in vitro* reagent may not be suitable for *in vivo* translational application and is associated with high toxicity. During the synthesis of Folate-PEG-PLL polymers, we produced various alternative formulations (Lipoplex) with surface modification of Folate-lipids in order to achieve: 1). high transfection efficiency 2). Low toxicity 3). Targeted gene delivery to macrophages 4). *In vivo* injectable formulation 5). simple manufacturing procedures.

Two different cationic liposomes composed of [N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) (DC-Chol/DOPE molar ratio 1:2) or Dimethyldioctadecylammonium bromide (DDAB) and cholesterol (DDAB/Chol, molar ratio 1:1) were made using the dry film method[6]. Briefly, all lipids were dissolved in ethanol. Surface modification were realized by adding either Folate-PEG-DSPE (targeting moiety, 2% molar percentage, PEG molecular weight 5kDa) or PEG-DSPE (non-targeting control, 2% molar percentage) in the mixture, and then the mixture were dried with N_2 gas. The dried thin films were rehydrated with DD water overnight, sonicated for 10 min and then passaged through 0.2μm syringe filter for 10 times. Effective diameter and zeta potential of liposomes were measured (Table 4).

Formulations	Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)
DC-Chol/DOPE(1:2) with PEG_{5k}-DSPE	46.2 ± 0.6	0.246±0.006	45.3±0.5
DDAB/Chol(1:2) with PEG_{5k}-DSPE	88.3±0.9	0.296±0.023	37.7±0.5
DC-Chol/DOPE(1:2) with Folate-PEG_{5k}-DSPE	202.4±32.1	0.426±0.066	29.9±0.2
DDAB/Chol(1:2) with Folate-PEG_{5k}-DSPE	239.8±27.1	0.454±0.048	36.5±1.3

Table 1. The effective diameter and zeta potential of cationic liposomes

The folate modified liposomes showed relative larger effective diameter and higher polydispersity which may require the optimization of folate-PEG-DSPE concentration. Lipoplex can be produced by gentle mixing calculated amount of DNA with cationic liposomes and keep at RT for 20 minutes. Uptakes and transfection efficiency can be determined by adding lipoplex into macrophage cells.

Cationic liposomes composed of Dimethyldioctadecylammonium bromide (DDAB) and cholesterol (molar ratio 1:1) as well as DDAB-Folate/Cholesterol were made. Their complexation with DNA and transfection efficiency were preliminarily determined by adding lipoplex (liposome/DNA complex; charge ratio 6:1) into human zmonocytes-derived macrophage cells (human blood monocytes treated with macrophage colony stimulation factor (MCSF) for 7days). These macrophage cells were grown in folate free RPMI1640 cell culture medium before adding lipoplex. Tomato red DNA was used to form lipoplexes and the expression of tomato red fluorescence protein was measured under confocal microscope everyday for up to 12 days. As shown in Figure 22, control (DNA alone) had no expression of tomato red while DC-Chol/DNA and DC-Chol-Folate/DNA formulations showed very low level of expression. On the contrary, postive control Geneporters efficiently transfected DNA to macrophage cells with peaking expression time at day 7. It is promising that DDAB/DNA and DDAB-Folate/DNA formulations exhibited comparable transfection efficiency to postive control but only postponed expression peak at day 10.

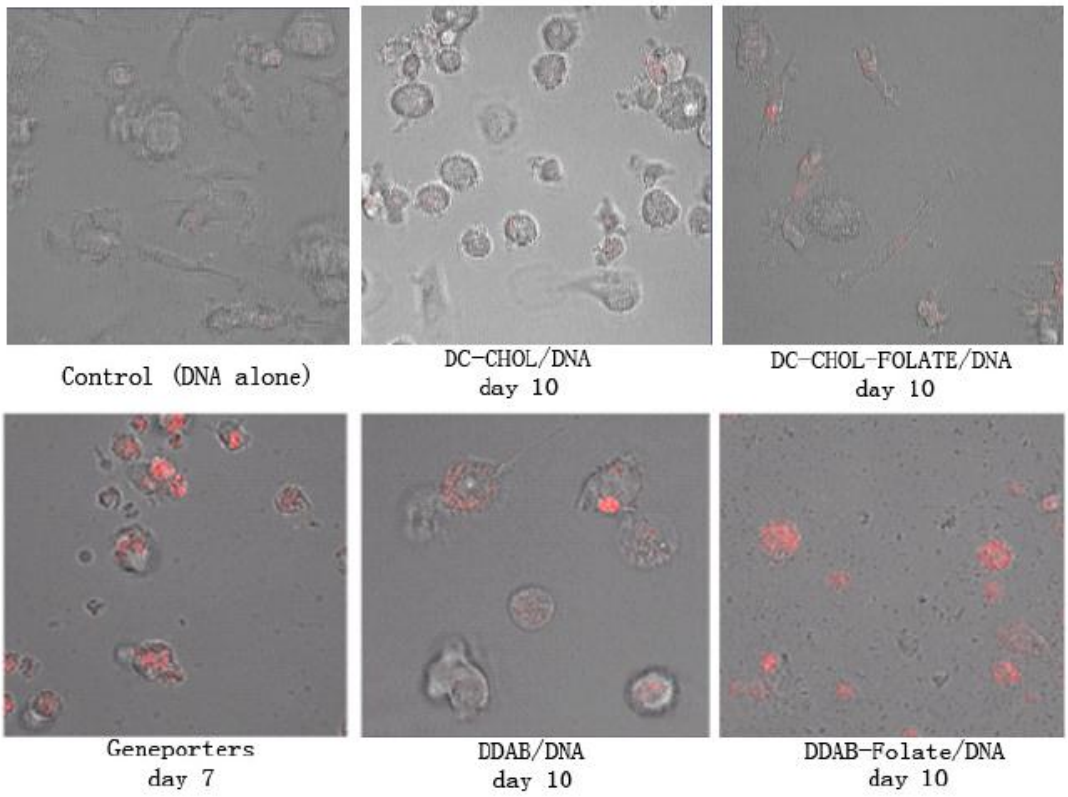


Figure 22. Tomato red protein expressions in human monocyte-derived macrophage cells

KEY RESEARCH ACCOMPLISHMENTS

- **Platform A:** Characteristics of polyanhydride nanoparticles capable to release stable full-length HA H5 protein were optimized in in vitro studies.
- **Platform A:** Initial studies on BALB/c mice subcutaneous and intranasal vaccinations with the soluble forms of the H5-T with and without different traditional adjuvantswere carried out These studies will be used to benchmark future work with nanoparticle-based adjuvants. The highest amount of neutralizing activity was observed in the groups receiving a Toll like receptor (TLR) agonist as an adjuvant. Only intranasal immunizations were able to elicit antigen specific IgA antibody in the bronchio-alveolar lavage fluid.
- **Platform B:** Following DNA-based vaccinations H5HA gene expressed, colocalized in APCs and translocated to the distal organs.
- **Platform B:** Pluronic/H5HA DNA vaccine formulations could induce lasting primary antibody response.
- **Platform B:** The mouse infectious dose of low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG) were determined in order to perform the challenge study.
- **Platform B:** Preinjection of Pluronic P85 (0.3% and 3% wt/wt) 36 hours before DNA injection in tibialis anterior muscle further increased gene expression compared to DNA alone and DNA+P85 without pre injection *in vivo*. These results demonstrate role of

infiltrating immune cells (neutrophils and macrophages) after preinjection and increasing transgene expression possibly by uptake of DNA and expression of transgenes in muscle tissue.

- **Platform B:** Increase in transgene expression *in vivo* was further confirmed by *in vitro* studies where co-incubation of plasmid DNA with increasing concentration of P85 with macrophages for 4 hours demonstrated increase in transgene expression. This i*n vitro* increase in transgene expression was optimal at 1% wt/wt P85, which was found to be up to 3 orders of magnitude compared to DNA alone.
- **Platform C:** Folate receptor expression was confirmed in RAW264.7 murine macrophage cells.
- **Platform C:** Alternative formulation 1-Folate-BICs were produced and cellular uptakes as well as in vitro transfection efficiency were preliminarily evaluated.
- **Platform C:** The synthesis of a new batch of cationic block copolymers Folate-PEG-PLL with higher purity (quality) is reaching the final click reaction procedure.
- **Platform C:** Alternative formulation 2-cationic liposome/DNA(lipoplexes) were produced, characterized and DDAB lipoplex showed promising transfection efficiency in human macrophage cells.

REPORTABLE OUTCOMES

Publications

1. AV Chavez-Santoscoy, LM Huntimer, AE Ramer-Tait, MJ Wannemuehler, and B Narasimhan, "Harvesting alveolar macrophages and evaluating cellular activation induced by polyanhydride nanoparticles," *J Vis Exp* (64), e3883, DOI:10.3791/3883 (2012)
2. Y Phanse, AE Ramer-Tait, SL Friend, B Carrillo-Conde, P Lueth, CJ Oster, GJ Phillips, B Narasimhan, MJ Wannemuehler, and MH Bellaire, "Analyzing cellular internalization of nanoparticles and bacteria by multi-spectral imaging flow cytometry," *J Vis Exp* (64), e3884, DOI:10.3791/3884 (2012)
3. Shaheen Ahmed, Tatiana K. Bronich, Alexander Kabanov. "Synthetic DNA Vaccine against Avian Influenza A Virus (H5N1)." *Molecular Therapy* **20**, Supplement 1(S96) (May 2012), the 15th Annual Meeting of the American Society of Gene & Cell Therapy, May 15-19, 2012, Philadelphia, PA, USA.

Conference Presentations

1. KA Ross*, L Huntimer, LK Petersen, MJ Wannemuehler, and B Narasimhan, "Stability and Release of H5N1 Hemagglutinin Antigens Encapsulated in Polyanhydride Nanoparticles," Annual AIChE Meeting, Minneapolis, MN, October 17, 2011

2. L Huntimer*, KA Ross, BR Carrillo-Conde, LK Petersen, AE Ramer-Tait, BH Bellaire, B Narasimhan, and MJ Wannemuehler, "Innate Immune Responses to Polyanhydride Nanoparticles Functionalized with a Toll Like Receptor Ligand," American Association of Immunologists Meeting, San Francisco, CA, May 14, 2011
3. Shaheen Ahmed, Tatiana K. Bronich, Alexander Kabanov. "Synthetic DNA Vaccine against Avian Influenza A Virus (H5N1)." The 15th Annual Meeting of the American Society of Gene & Cell Therapy, May 15-19, 2012, Philadelphia, PA, USA.
4. KA Ross*, L Huntimer, LK Petersen, MJ Wannemuehler, and B Narasimhan, "Stability and Release of H5N1 Hemagglutinin Antigens Encapsulated in Polyanhydride Nanoparticles," Annual AIChE Meeting, Minneapolis, MN, October 17, 2011
5. V. Mahajan, A. Kabanov, "Pluronic block copolymer as a potential gene delivery candidate", 15th Annual meeting of American Society of Cell and Gene Therapy (ASGCT), Philadelphia, PA, May 15 -19 2012.

CONCLUSIONS

- In response to administration of pVAX1-H5HA DNA and pVAX1-H5HA DNA/P85 or SP1017 the APC incorporated, expressed and transported the H5HA transgene to the central immune organs. Pluronic/pVAX1-H5HA DNA vaccine formulations could induce a primary antibody response and that the level of the induced total IgG antibody was long lasting.

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